Streptococcus *pneumoniae* colonisation in a cohort of SE Asian infants

Thesis

Streptococcus pneumoniae Colonisation in a Cohort of SE Asian Infants

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Abstract

Background

Streptococcus pneumoniae is a leading cause of childhood mortality and morbidity. Nasopharyngeal colonisation precedes infection but the dynamics, modifiers, and immunological outcomes of colonisation in infancy are incompletely understood.

Methods

We conducted a longitudinal pneumococcal colonisation study of mothers and infants in Maela refugee camp in NW Thailand. 965 infants were followed from birth until 24 months.

Results

Pneumococcal colonisation occurred early in infancy (median 46d). Vaccine serotypes (PCV13) accounted for 55.8% of isolates from infants and 27.5% from mothers. Non-typeable pneumococcal colonisation was common. Previous colonisation did not result in protection against subsequent pneumococcal acquisitions in infancy, although serotype reacquisitions tended to be delayed and of shorter duration.

Positive associations were found between colonisation by pneumococci and *Haemophilus influenzae* (OR 2.9, $P<.001$) or *Moraxella catarrhalis* (OR 2.1, $P<.001$), whereas *Staphylococcus aureus* colonisation was negatively associated (OR 0.3, $P<.001$). During first pneumonia episodes, detection of respiratory syncytial virus was negatively associated with pneumococcal colonisation (OR 0.5, $P=.02$).

Serum IgG anti-capsular antibody responses to colonisation varied by capsule and increased with age: these antibodies were not protective against colonisation in infancy. Although cord bloods had high titres of IgG to pneumococcal surface and virulence proteins, none were associated with delayed infant colonisation. These proteins were immunogenic in infants but antibodies did not protect against colonisation.

Latex sweep serotyping was 3.9 times, and microarray 4.4 times, more likely to detect multiple pneumococcal serotype co-colonisations than standard WHO protocol culture ($P<.001$).

Conclusions

Pneumococcal colonisation is influenced by previous exposure to homologous and heterologous serotypes. Neither previous exposure nor serum antibodies to capsule or surface proteins protect against colonisation in the first 24 months of life. WHO methodology significantly underestimates multiple serotype colonisations. These colonisation data will be a valuable baseline to compare with future studies following regional introduction of pneumococcal conjugate vaccines.

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List of abbreviations

AdV	Adenovirus
ANC	Antenatal clinic
AOM	Acute otitis media
ARI	Acute respiratory infection
CC	Clonal complex
CCSDPT	Committee for Coordination of Services to Displaced Persons in Thailand
CDC	US Centers for Disease Control and Prevention
CI	Confidence interval
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
CRF	Case record form
CoNS	Coagulase negative staphylococcus
CNA	Colistin-nalidixic acid agar
cT	Cycle threshold
d	Days
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Program on Immunisations
Flu	Influenza virus
GAVI	Global Alliance for Vaccines and Immunisation
GMC/T	Geometric mean concentration / titre
GPS	Global Positioning System
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
hMPV	Human metapneumovirus

HR	Hazard ratio
Ig	Immunoglobulin
ILI	Influenza-like illness
IMCI	Integrated Management of Childhood Illness
IPD	Invasive pneumococcal disease
IQR	Inter-quartile range
km	Kilometre
LRTI	Lower respiratory tract infection
µg	Microgram
µL	Microlitre
m	Months
mL	Millilitre
mm	Millimetre
MIC	Minimum inhibitory concentration
MLSA	Multi-locus sequence analysis
MLST	Multi-locus sequence typing
MORU	Mahidol-Oxford Tropical Medicine Research Unit
NA	Not applicable
ND	No data
NGO	Non-governmental organisation
NPA	Nasopharyngeal aspirate
NPS	Nasopharyngeal swab
NT	Non-typeable
NVT	Non-vaccine type
OR	Odds ratio
PCR	Polymerase chain reaction

PCV	Pneumococcal conjugate vaccine
Pnc	Pneumococcus / *Streptococcus pneumoniae*
PU-AMI	Premiere Urgence-Aide Médicale Internationale
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNaseP	Ribonuclease P
RSV	Respiratory syncytial virus
rRT-PCR	Real-time reverse transcription PCR
SMRU	Shoklo Malaria Research Unit
SOP	Standard operating procedure
ST	Sequence type
STGG	Skimmed milk, tryptone, glucose, glycerol medium
TBBC	Thai Burmese Border Consortium
UNICEF	United Nations Children's Fund
URTI	Upper respiratory tract infection
VT	Vaccine type
VTM	Viral transport medium
WHO	World Health Organisation
y	Years

1 Background and Introduction

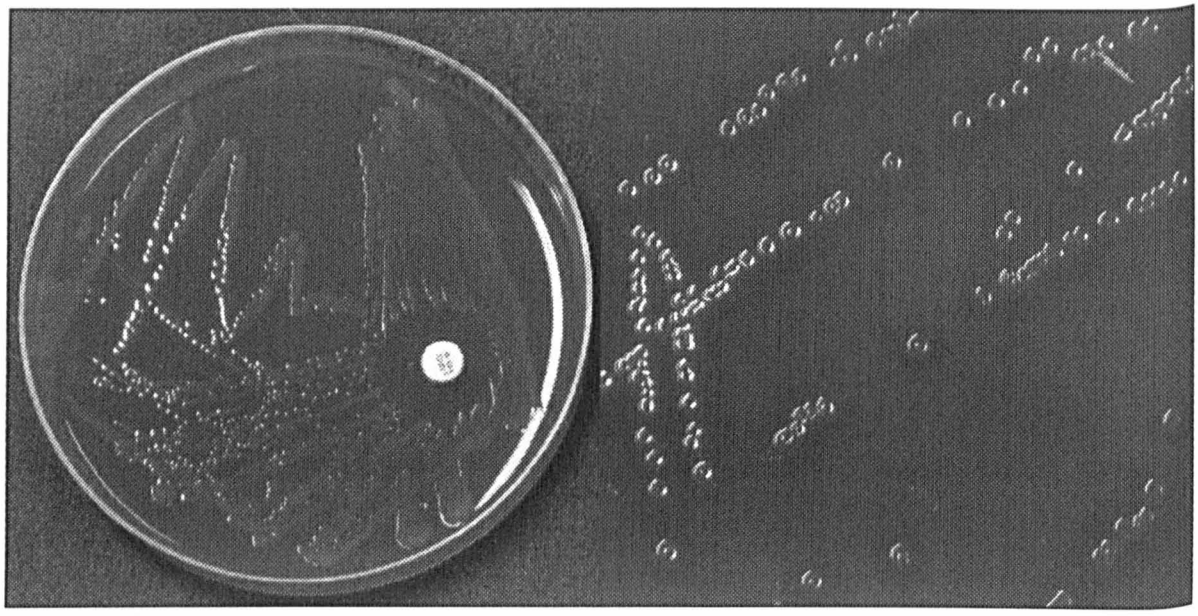
1.1. Streptococcus pneumoniae

Streptococcus pneumoniae (the pneumococcus) is a leading infectious cause of morbidity and mortality. First identified independently by Pasteur and Sternberg in 1881, the organism rapidly became acknowledged as an important cause of lobar pneumonia (1). Pneumococcal disease ranges from mild mucosal infection, such as conjunctivitis and acute otitis media (AOM), to severe systemic infection, such as bacteraemic pneumonia and meningitis. The organism has been estimated to cause around 10% of all deaths in HIV-negative under five year olds: a total of 826,000 deaths in the year 2000 (2). The majority of these deaths occur in Africa and Asia. In recent years, the introduction of pneumococcal conjugate vaccines (PCV) has resulted in great reductions in invasive pneumococcal disease due to the 7 – 13 serotypes covered by the vaccines (3-6).

Streptococcus pneumoniae is a catalase-negative, alpha-haemolytic Gram positive diplococcus (Figure 1). *S. pneumoniae* is closely related to the upper respiratory tract commensals *Streptococcus infantis, Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus pseudopneumoniae* (collectively known as the Mitis group streptococci). Recent genetic analyses by Kilian and colleagues demonstrated that these species arose from a common pathogenic ancestor and that *S. pneumoniae* represents one lineage in the overall pneumoniae-pseudopneumoniae-mitis cluster. This cluster evolved into distinct lineages of commensal species as a result of habitat adaptation by virulence gene loss (7). Exchange of genetic material within *S. pneumoniae,* and between species of the larger pneumoniae-pseudopneumoniae-mitis cluster, is the principal pneumococcal evolutionary mechanism (8). *S. pneumoniae* can be phenotypically differentiated from the other members of the Mitis group of streptococci by its susceptibility to optochin (ethyl hydrocuprein hydrochloride) and bile solubility (sodium deoxycholate), although atypical pneumococci may be optochin resistant or bile insoluble (9). The species may be

distinguished from its closest relatives by comparison of sequences seven housekeeping genes using multilocus sequence analysis (eMLSA; http://www.eMLSA.net) (10).

Figure 1. Pneumococcal culture plate and colony morphology



1.2. Typing of *Streptococcus pneumoniae*

1.2.1. Serotyping

The polysaccharide capsule forms the basis of the major typing system for pneumococci. In all but two serotypes, the capsule is synthesised by a Wzx/Wzy-dependent pathway encoded by the genes of the capsular polysaccharide synthesis (*cps*) locus (11, 12). A serotype-specific series of genes are responsible for the synthesis of the capsule polysaccharide subunit, polymerisation of this subunit, and subsequent translocation to the cell surface. The capsules of serotypes 3 and 37 are synthesised by an alternative synthase pathway. Isolates may be serotyped on the basis of reactions to specific anti-capsule antisera. The antisera are raised in rabbits (Statens Serum Institut, Hillerod, Denmark) and are divided into pool, group, type, and factor antisera allowing serotyping to be carried out in a checkerboard scheme (Table 1 & Table 2). Using the Danish numbering system, serotypes are organised into 46 serogroups based on anti-sera

cross-reactivity, and then separated into serotypes by unique antisera reactions. Individual serotypes within a larger serogroup are denoted by a letter suffix (e.g. 19F, denoting the first serotype identified with serogroup 19). An alternative American typing scheme, which numbered serotypes in order of their discovery, has lost favour to the Danish system (13, 14).

Table 1. Pneumococcal serotyping using pool and type antisera

Pool	Vaccine groups/types					Non-vaccine groups/types
	P	Q	R	S	T	
A	1	18*	4	5	2	
B	19*	6*	3	8		
C	7*				20	24*, 31, 40
D			9*		11*	16*, 36, 37
E			12*	10*	33*	21, 39
F				17*	22*	27, 32*, 41*
G						29, 34, 35*, 42, 47*
H	14	23*		15*		13, 28*
I						25*, 38, 43, 44, 45, 46, 48

*Indicates that there are >1 serotypes within the serogroup

Table 2. Resolution of serogroup 19 into distinct serotypes using factor antisera

Serotype	Factor antisera			
	19b	19c	19f	7h
19F	+	-	-	-
19A	-	+	-	-
19B	-	-	-	+
19C	-	-	+	+

Observation of capsular swelling by microscopy (the Quellung reaction) has been the preferred method of typing pneumococci since its description by Neufeld at the beginning of the 20th century (15). However, this method of typing is costly and time consuming. Alternative methods, including counterimmunoelectrophoresis (16), dot blot (17), and slide or latex agglutination techniques (18-21), have been developed to improve

the ease of typing pneumococci. A recent review of European pneumococcal reference laboratories found an overall serotyping error rate of 5% and concluded that there was no association between typing method (Quellung vs. non-Quellung) and error rate (22).

Sequencing of the capsule biosynthesis locus (*cps*) for all known pneumococcal serotypes opened the door for the development of molecular serotyping tools (11). Methods to identify up to 40 serotypes by either conventional or real-time polymerase chain reaction (PCR) have been described (23-25). PCR of the entire *cps* locus, followed by RFLP (restriction fragment length polymorphism) analysis and comparison to a database of serotype-specific band patterns, or PCR and sequencing of the regulatory *cpsB* gene may also be used to determine pneumococcal serotypes (26, 27). A combination of multiplexed immunoassay and PCR, with a Luminex microsphere-based detection system, has been developed to detect all known pneumococcal serotypes and non-typeable pneumococci (28). However, to date, microarray-based typing has been the only molecular tool to be able to detect all known pneumococcal serotypes in a single reaction (29). These methods are discussed further in the section on multiple serotype colonisation (section 1.8.1).

1.2.2. Genotyping

Due to the organism's ability to undergo genetic recombination, genotyping offers increased resolution over serotyping to study pneumococcal population structures and strain specific characteristics. Multi-locus sequence typing (MLST) is currently the standard genotyping technique for *S. pneumoniae*, although this will inevitably change as the cost of whole genome sequencing falls (30). MLST involves PCR amplification and sequencing of seven pneumococcal housekeeping genes and subsequent assignment of a numeric "sequence type" (ST) based on these sequences using the MLST website (http://spneumoniae.mlst.net/). The numeric nature of the sequence type, coupled with the non-ambiguity of sequence data as compared with gel images used in alternative typing

schemes such as pulsed field gel electrophoresis (PFGE), permits comparisons of MLST data globally. Relationships between STs can be determined using computer algorithms such as eBURST (http://spneumoniae.mlst.net/eburst/) (31). As an example of the utility of characterisation of isolates by MLST compared to serotyping alone, an analysis of colonising pneumococci in Tanzanian children revealed nasopharyngeal co-colonisation by multiple genotypes of the same serotype (32).

However, there are limitations to the current MLST scheme. Analysis of strain collections using either whole genome sequencing or an expanded 96-locus MLST scheme has revealed the considerable genetic diversity within single 7-locus STs (33, 34). Genetic recombination may result in the acquisition, loss or variation in key virulence determinants within a lineage (8). Croucher et al. identified >700 recombination events in a whole genome sequencing study of 240 isolates of the globally successful pneumococcal clone PMEN1 (ST81) collected over a 24 year period. Recombination resulted in capsule switches on ten occasions, leading to on-going success of the clone despite PCV7 introduction (33).

1.3. Antimicrobial resistance

Resistant isolates of *S. pneumoniae* have become increasingly prevalent (35), with a number of drug-resistant clones having spread internationally (33, 36, 37).

Beta-lactam resistance in pneumococci occurs as a result of expression of mosaic *pbp* genes. These genes encode penicillin binding proteins (PBPs) with reduced affinity for beta-lactam molecules. Mutations in PBP1a, PBP2b, and PBP2x account for the majority of observed resistance. Point mutations occur in *pbp* genes in closely related streptococci and blocks of these genes may be subsequently acquired by pneumococci by horizontal gene transfer (38). Macrolide resistance is generally disseminated via transposons (33, 38). Acquisition and expression of the *erm*(B) gene results in modification of the bacterial 23S ribosomal subunit with a reduction in macrolide binding affinity. Efflux pumps, coded by

mef genes, are an alternative macrolide resistance mechanism in pneumococci. Non-typeable pneumococci may be a significant reservoir for antimicrobial resistance genes (39).

A significant proportion of pneumococcal isolates from Asian children have been found to be antimicrobial resistant (40). Resistant organisms are largely confined to the types which commonly colonise children (serogroups 6, 14, 19, and 23). Residence in urban areas, day-care attendance, and previous history of otitis media have been shown to be risk factors for carriage of non-susceptible pneumococci by Asian children (40). Several studies have demonstrated significant levels of resistance in Thai pneumococcal isolates (41, 42). A recent six year review of 115 invasive pneumococcal isolates from Thai children demonstrated that 69.6% were non-susceptible to penicillin (26.1% were intermediate and 43.5% were resistant) (43). Eighty nine percent of these non-susceptible isolates were of serotypes contained in the seven valent conjugate vaccine.

A significant additional benefit associated with the widespread use of the pneumococcal conjugate vaccine is a reduction in the burden of invasive pneumococcal disease due to antimicrobial resistant strains, although over time this may be eroded by the increasing resistance in non-vaccine serotypes (44, 45).

1.4. Pneumococcal colonisation and disease

Colonisation of the nasopharynx by *S. pneumoniae* ("pneumococcal carriage") is believed to be essential for subsequent infection (46). A longitudinal study of infants from Alabama demonstrated that nasopharyngeal acquisition of a pneumococcal serotype was followed, within one month, by infection (mostly AOM) in 15% of cases (47). Invasive pneumococcal serotypes were found more frequently in nasal specimens at lower respiratory infection sampling points than at healthy sampling points in a cohort of Papua New Guinean children (48). Due to the inherent difficulties of obtaining invasive disease

isolates, nasopharyngeal colonisation data have been used to determine the likely serotypes and antimicrobial resistance of pneumococci causing invasive disease (49-52).

Individual serotypes have different disease potentials, and this appears to be determined by both capsule and other genetic determinants. In Papua New Guinean children, Smith et al. found that invasion was more related to acquisition than persistence in the nasopharynx. Certain serotypes, notably 1, 5, and 46, were found to be highly invasive but rarely isolated from nasopharyngeal specimens (53). Brueggeman and colleagues compared invasive and carriage isolates in the UK and found that the most invasive serotypes (1, 5, and serogroup 7) were the least frequently carried and the least invasive serotypes (serogroups 6, 19, and 23) were the most frequently carried (54). The same group also determined that there was little variability in invasive potential amongst different clones (as defined by MLST) of the same serotype, but that there was considerable variability in the invasiveness of different serotypes of the same MLST genotype (55). Similar results were reported from Finland (56). Interestingly Sandgren et al. determined that Swedish pneumococcal strains of the same serotype, but different MLST genotype, had different invasive potentials suggesting that the capsule is not the sole determinant of invasiveness (57).

There are also temporal and geographic trends in serotypes resulting in invasive infections (58). For example, invasive infections due to serotype 2 were noted to disappear from Boston in 1967 but this serotype has recently emerged as the commonest meningitis-associated serotype in Bangladesh (59, 60). Feikin and Klugman reviewed several invasive pneumococcal disease datasets from the USA, covering the period 1928 – 1998. In both adults and children, they found a significant decline in the proportion of infections caused by the so-called "epidemic" serotypes (1 – 3 and 5) and a significant increase in infections by the PCV7 serogroup/types (4, 6, 9, 14, 18, 19, and 23). The authors concluded that changes in sampling (a trend towards lower thresholds for taking blood cultures over time), antimicrobial prescribing practices, socioeconomic conditions, and population-level

immunocompromise (due to an ageing population and the arrival of HIV infection) could account for this shift (61). Scott et al. reviewed over 7,000 episodes of invasive pneumococcal infection from 13 existing datasets and determined that disease due to serotypes 1 and 5 was more common in South America compared with the other geographic regions included in the analysis. Clear differences in the age distribution of disease caused by individual serotypes were found: risk of serotype 1 disease declined progressively through life and the opposite was true for serotype 3 (62). Similar results were described by Hausdorff and colleagues in an analysis of global vaccine coverage (63). The proportion of invasive pneumococcal disease caused by PCV7 serogroups was found to vary by geographic area (significantly lower in South America and Asia) and age group (lower coverage in older children and adults). This paper highlighted the relatively small dataset from the Asian region from which conclusions regarding invasive pneumococcal disease could be made. This point has been reinforced in several subsequent reviews (2, 64, 65). In the most recent summary of South East Asian data, Jauneikaite et al. concluded that the most common serotypes associated with invasive disease were 19F, 23F, 14, 6B, 1, 19A, and 3. Of note, several of the poorest countries from the region contributed either limited or no data, for example Laos, Cambodia, or Myanmar (66).

PCVs reduce colonisation by serotypes covered by the vaccine (67). Non-vaccine serotype colonisation increases as a result of this, leading to an increase in NVT invasive disease (68). Therefore, studies of nasopharyngeal colonisation are an important component of surveillance, both pre- and post-pneumococcal vaccine introduction (69).

1.5. Pneumococcal colonisation dynamics in early childhood

Streptococcus pneumoniae is considered part of the normal human nasopharyngeal flora and colonisation is particularly common in the young. There have been many studies of pneumococcal colonisation (summarised in (70) and (46)), and the following discussion focuses on studies from World Bank defined low-income countries from Asia and Africa,

comparing with studies from other countries and regions where appropriate. Cross-sectional studies including serotype prevalence meeting these criteria and all longitudinal carriage studies commencing at or around birth are summarised in Table 3 and Table 4.

1.5.1. Acquisitions

In developing world populations nasopharyngeal acquisition of pneumococci occurs very early in life. Studies in Papua New Guinea have documented a median age at acquisition of 19 days and universal colonisation by three months of age (71, 72). Similar studies in The Gambia found a median age of acquisition of 24 days and universal colonisation within the first year of life (73). A recent intensive study of 1,404 Kenyan infants over the first three months of life showed a median time to acquisition of 38.5 days, with a pneumococcal acquisition rate of 0.0189 (95% CI 0.0177 – 0.0202) per day (74). In the past, similar pneumococcal acquisition rates were observed in high-income country infants. For example, 15.2% of a cohort of 99 Glaswegian infants born in 1950 were colonised within the first week of life and 62.6% had been colonised at least once by 47 weeks of age (75). In the 1970s, Gray and co-workers demonstrated a mean age at pneumococcal acquisition of six months and near universal colonisation within the first two years of life in a cohort of 82 Alabaman infants (47). More recent studies have shown more modest acquisition rates. In the Finnish infant cohort study, 11% of infants had been colonised by two months, rising to 56% by 12 months and 87% by 24 months (76).

Following primary acquisition in infancy, frequent re-acquisition of pneumococci may occur. In a longitudinal study of children aged six weeks to six years in a US day-care centre, children followed for more than 24 months carried between two and 12 pneumococcal serotypes. Re-acquisition of the same serotype was not uncommon, and up to four reacquisitions of an individual serotype were documented (77). Hill et al. documented 1,145 episodes of carriage in 236 Gambian infants followed over the first year of life: 909 episodes occurred after clearance of the first acquired pneumococcus (73).

1.5.2. Carriage duration

Carriage duration varies by both serotype and age, although methodological differences between studies including definitions of a serotype "carriage episode" can make direct comparison difficult. However, some consistent observations have been made. Certain serotypes, notably 1 and 5, are carried for very short durations and are therefore rarely isolated from the nasopharynx despite being responsible for a significant proportion of invasive disease (53, 78, 79). Others, notably serogroups 6, 19, and 23, may be carried for long periods (47, 53, 72, 73, 77, 78). Capsule size has been found to correlate with carriage prevalence (80). Larger capsules are more resistant to neutrophil phagocytosis, which may result in prolonged carriage duration (81).

1.5.3. Risk factors for colonisation

Several factors have been associated with increased rates of pneumococcal carriage in children: age <2 years, overcrowding, antibiotic use, day-care attendance, and parental smoking (51, 82, 83). Pneumococci are transmitted primarily via respiratory droplets, which is likely to be particularly effective in crowded homes or day-care centres and may be enhanced by coryza (74, 79, 84). A study of carriage in Southern Vietnam determined that children living in rural areas were more likely to be carriers than those living in urban and suburban areas (85). Interestingly, a study from Hong Kong found that the carriage rate of *S. pneumoniae* was significantly higher in ethnic Vietnamese compared to Chinese children (86). Traditional risk factors for colonisation, namely age, overcrowding, and parental smoking, could not explain this difference. Differences in carriage prevalence by ethnic group were not found in The Gambia or Nigeria (87, 88). In Western Australia, Aboriginal children were colonised by pneumococci considerably earlier than non-Aboriginal children living in the same geographic area. By six months, 76% of Aboriginal and 47% of non-Aboriginal children had been colonised (89).

Table 3. Cross-sectional studies of pneumococcal colonisation and serotype distribution in children from World Bank defined low-income African and Asian countries

Country	Year	Location	N	Age group	Health status	Specimen	% colonised	% PCV7 serotypes	% non-typeable	Commonest five serotype/groups	Reference
Bangladesh	1999 – 2000	Mixed	2,839	<5y	Healthy	Nasal swab	46	ND	ND	6, 9, 15, 23, 16	(90)
CAR*	1997	Urban	630	2 – 59m	Out-patient visits	NPS	60	47	ND	19F, 6B, 6A, 14, 23B	(91)
China	1999	Urban	269	<5y	Out-patient visits	Nasal swab	37	ND	21	19, 6, 14, 23, 17	(92)
China	1999 – 2005	Urban	2,425	<5y	Unwell (URTI)	NPS	27	ND	ND	19, 23, 6, 14, 15	(93)
Fiji	2003 – 4	Mixed	774	3 – 13m	Healthy	NPS	44	31	4	6A, 23F, 19F, 6B	(94)
Gambia	1989 – 91	Rural	113	<5y	Healthy	NPS	76	ND	0	19, 6, 23, 9, 15	(95)
India	Pub. 2007	Urban	200	3m – 3y	Out-patient visits	NPS	7	85	8	1, 6, 14, 19, NT	(96)
Indonesia	1997	Mixed	484	0 – 25m	Healthy	NPS	48	61	11	6, 23, NT, 15, 33	(97)
Kenya[a]	2004	Mixed	349	0 – 4y	Community – all	NPS	57	47	1	19F, 6B, 6A, 23F, 14	(98)
Malawi	1997	Rural	906	2 – 59m	Out-patient visits	NPS	84	43	0	6A, 19A, 23F, 19F	(99)
Mozambique	2003	Rural	285	<5y	Out-patient visits	NPS	87	49	5	19F, 19A, 23F, 6A, 6B	(100)
Nepal	2003 – 4	Rural	1,100	1 – 36m	Healthy & unwell (ARI)	NPS	79	58	ND	6, 19, 23, 15, 9	(101)
Nigeria[a]	Pub. 2012	Peri-urban	375	<5y	Community – all	NPS	71	46	3[b]	19F, 6A, 6B, 23F, 15B	(88)
Thailand[a]	2002 – 4	Rural	~425	<5y	Unwell (ILI/pneumonia)	NPS	60	55	11[b]	ND	(102)
Vietnam[a]	Pub. 2000	Mixed	389	<5y	Community – all	Nasal swab	49	ND	6[b]	14, 19, and 23	(103)
Vietnam[a]	2003 – 4	Mixed	722	<5y	Community – all	Nasal swab	47	ND	20[b]	19, 23, NT, 6, 14	(85)
Zambia	1994	Rural	260	<6y	Out-patient visits	NPS	72	ND	ND	ND	(104)

*Central Asian Republics

[a] Studies also included children ≥5y and/or adults

[b] Not restricted to isolates from children <5y

Table 4. Longitudinal studies of pneumococcal colonisation from birth

Country	Year	Location	N	Follow-up period	Swabs (/infant)	Swab interval	Pneumococcal acquisition	Carriage duration?	% PCV7 serotypes	% non-typeable	Commonest five serotype/groups	Reference
Australia	1992 – 3	Rural	50	0 – 270d	ND	2 – 4w	Median age 58d >90% by 120d (Aboriginal data)	No	ND	ND	ND	(105)
Bangladesh	2000 – 1	Rural	99	0 – 12m	16	Varied	50% by 8w 90% by 21w 100% by 12m	No	ND	3	6, 19,15, 23, 10	(106)
Costa Rica	1988 – 92	Mixed	440	0 – 12m	4 or 52	3m or 1w	95 – 100% by 1y	No	ND	ND	ND	(107)
Finland	1994 – 5	Urban	329	2 – 24m	10	Varied	11% by 2m 56% by 12m 87% by 24m	No	53	5	6B, 23F, 19F, 6A, 11	(76)
Gambia	Pub. 2008	Rural	236	0 – 12m	16	Varied	Median age 24d 100% by 12m	Yes	46	ND	6B, 19F, 6A, 14, 23F	(73)
Gambia	Pub. 2010	Rural	196	0 – 12m	4	Varied	98% by 12m	Yes	43	ND	19F, 6A, 6B, 14, 23F	(108)
India	1994 – 5	Not stated	100	0 – 18m	7	Varied	Median age 11w 81% by 18m	Yes	ND	ND	6, 19, 14, 15, 23	(109)
India	1998 – 9	Rural	464	0 – 6m	3	2m	54% by 2m 86% by 6m	No	ND	ND	23, 19, 6, 15,14	(110)
Kenya	2006 – 9	Mixed	1404	0 – 13w	≤15	Varied	Median age 38.5d Rate 0.0189 / day	No	36	0	19F, 6A, 6B, 23F, 23B	(74)
Papua New Guinea	Pub. 1986	Rural	25	0 – ≤10m	ND	5 – 14d	60% by 15d 100% by 3m	Yes	ND	ND	ND	(72)
Philippines	Pub. 2006	Not stated	173	0 – 10m	6	Varied	50% by 10w 90% by 10m	No	ND	ND	ND	(111)

Country	Year	Location	N	Follow-up period	Swabs (/infant)	Swab interval	Pneumococcal acquisition	Carriage duration?	% PCV7 serotypes	% non-typeable	Commonest five serotype/groups	Reference
Sweden	1985 – 8	Urban	468	0 – 10 or 18m	3 or 4	4m	11% by 2m 34% by 4m 48% by 10m	No	ND	ND	ND	(112)
UK	Pub. 1999	Urban	72	0 – 6/18m	7	1m	ND	No	ND	ND	ND	(113)
UK	1999 – 2001	Not stated	213	0 – 24w	9	Varied	15% by 4w 48% by 12w 54% by 24w	Yes	56	0	6B, 19F, 23F, 14, 6A	(78, 114)
USA	1974 – 5	Urban	82	0 – 24m	12	Varied	Mean age 6m 96% by 24m	Yes	ND	1	6, 19, 23, 14, 9	(47)
Zambia	2003 – 4	Peri-urban	128	0 – 18m	7	3m	No acquisition data 26% swabs positive	No	44	6	19F, 6B, 23F, 15, 14	(115)

1.6. Colonisation in older individuals

Cross-sectional studies have documented a fall in pneumococcal colonisation prevalence with increasing age. In Kenya, pneumococcal carriage prevalence was 57% for 0 – 4 year olds, 41% for 5 – 9 year olds, and 6.4% for 10 – 85 year olds (98). Similar trends were seen in Nigeria and The Gambia, although adult carriage prevalence was significantly higher (87, 88). In Sa Kaeo province on the Eastern border of Thailand, carriage prevalence fell from 60% in children under five years to 16% in adults fifty years or older (102). In the UK, colonisation prevalence was 52% in children under two years and 8% in adults (≥18 years) in a longitudinal household study (116).

Carriage duration has also been shown to decrease with increasing age. In the Gambian village study, the mean duration of carriage decreased from 27.9 weeks for children aged <1 year to 2.9 weeks in adults aged ≥40 years (79). Similar findings were documented in studies of carriage of penicillin-resistant pneumococci in Sweden (117, 118).

The development of mucosal immunity and less frequent exposure are potential contributors to this age-related decline in colonisation and carriage duration (119-121).

1.7. Non-typeable pneumococci and colonisation

Pneumococci that fail to type with anti-sera may be either non-encapsulated "rough" strains as a result of deletion or disruption of the *cps* locus, encapsulated strains with intermittent or low level capsule expression, or encapsulated strains of a novel capsular type (122). Early in the 20th century Griffiths demonstrated that encapsulated pneumococci could become rough on prolonged or repeated sub-culture on solid media (123).

A detailed description of rough pneumococci was given by Paul in 1927 (124). At this time, it had already been recognised that these organisms were non-encapsulated pneumococcal forms of low virulence, although they could be isolated from the sputum of

pneumonia cases (125). More recent publications have found that these organisms can be associated with outbreaks of mucosal disease, especially conjunctivitis (126-132). However, it has recently be confirmed that non-typeable (NT) pneumococci, either due to absent or down-regulated capsular biosynthesis genes, can also cause invasive infection (133).

Several studies have determined that non-typeable pneumococci may be either genetically "typical" pneumococci or more "atypical" organisms, clustering outside of the *S. pneumoniae* group and closer to *S. mitis* (9, 134). Isolates of the recently described species *S. pseudopneumoniae* may also be misidentified as NT pneumococci (135). As has been the case for typical encapsulated pneumococci, globally successful NT lineages have been identified (e.g. ST344 and ST448) (131, 136).

The *cps* locus in NT pneumococci may be disrupted by the presence of either an *aliB*-type locus (referred to as *aliC / aliD* in reference (137)) or the presence of a novel surface protein gene (*nspA / pspK*) (137-139). On the basis of predicted structure and murine colonisation model data, the novel surface protein gene may be required for successful colonisation of the nasopharynx.

It is hard to fully assess the prevalence of these organisms from carriage studies, since they are not consistently identified or reported. Additionally, with the increasing reliance on molecular serotyping tools which mostly cannot identify all known serotypes, the reported prevalence of PCR non-typeable pneumococci may not equate to the prevalence of antisera non-typeable pneumococci. The percentage of pneumococci that were non-typeable ranged from 0 – 21% in the studies described in Table 3 and a study of Spanish primary school children found 44.5% of carried pneumococci were non-typeable (140). Simoes et al. showed that the detection of NT pneumococcal colonisation increased three-fold (from 2.9% to 8.6% of nasopharyngeal specimens), when a multiplex-PCR assay designed specifically to identify NT pneumococci was compared with culture of Portuguese nasopharyngeal specimens by the WHO (World Health Organisation) protocol

(141). Using a combination of multiplex PCR and microarray, Brugger et al. determined that NT pneumococci were more frequently identified in individuals as a co-coloniser rather than as the only colonising pneumococcal "type" (142). Marsh et al. determined that non-typeable pneumococcal carriage rates were 5 – 20% in Indigenous Australian children, and that these organisms were frequently drug resistant (39). It has been shown that NT pneumococci may act as a reservoir for drug resistance genes which may be transferred to encapsulated pneumococcal strains (143).

1.8. Colonisation by multiple pneumococcal serotypes

It has long been appreciated that multiple pneumococcal serotypes may co-exist in the nasopharynx. Using mouse inoculation, it was found that 14% of nasopharyngeal swabs from families of pneumonia cases contained >1 pneumococcal serotype and that the prevalence of multiple serotype carriage was highest in children (144). Gratten et al. serotyped up to six colonies from nasal swab culture plates and found multiple serotype carriage in 29.5% of Papua New Guinean children (145). Using multiplex PCR, a second serotype was found in 20% of pneumococcal colonised Waro Amerindian Children (146).

1.8.1. Detection of multiple serotype colonisation

Detection of multiple pneumococcal serotype colonisation is challenging. Early work relied on mouse inoculation and was very labour intensive (144). Using agar-based culture, colony morphology rarely distinguishes between pneumococcal serotypes with a few exceptions (e.g. serotype 3 and NT pneumococci). Work from Papua New Guinea determined that multiple serotypes were not present in equal abundance in the nasopharynx. By serotyping 50 individual colonies from nasal swab cultures, Gratten and colleagues found that a minor serotype was present at a relative abundance of 4 – 27% in 5/10 specimens (145). Brugger et al. confirmed this result by *ply* PCR and terminal-RFLP analysis. They found a median ratio of serotypes of 1:3.8 (range 1:1 – 1:45) in 41 co-

colonised individuals, i.e. the minority serotype was present at a median 26% (range 2 – 50%) relative abundance (142).

It would not be practical, or cost-effective, to serotype large numbers of colonies to determine multiple serotype colonisation in a large carriage study. It has been estimated that, to reliably detect a minor serotype of 25% relative abundance, 11 colonies would need to be serotyped and this rises to 59 colonies in order to detect a serotype at a relative abundance of 5% (147). An Australian study concluded that picking four colonies for serotyping by random selection was more effective at identifying multiple serotype colonisation than picking colonies on the basis of morphological differences (17% vs. 14% of specimens) (148).

Several techniques have been developed to improve the detection of multiple serotype carriage. A colony blot method was described by Bogaert et al., which could detect multiple serotypes in the ratio of 1:1000 (149). Bronsdon and colleagues described a similar method, but theirs was limited to the detection of a small number of serotypes (150). Researchers in The Gambia developed a latex agglutination technique where a sweep of colonies from the primary nasopharyngeal swab (NPS) culture plate were suspended in saline and serotyped by latex agglutination. Using this method in a longitudinal infant cohort study, up to 10.4% of pneumococcal acquisitions were found to be acquisitions of multiple serotypes (73). A broth enrichment step followed by either conventional Quellung typing or multiplex PCR improved both detection of pneumococci and multiple serotype carriage in studies from Denmark and the USA (25, 151). However, recent data from CDC suggest that the molecular approach may have flawed specificity due to the presence of pneumococcal *cps* locus homologs in closely related streptococcal species including *S. oralis* (152). A combined Luminex-based multiplex immunoassay and PCR has been demonstrated to be able to correctly identify multiple serotypes in laboratory generated mixed cultures, down to a relative abundance of 10% and perhaps lower (28). Recently, progress has been made in the detection of multiple serotype colonisation

directly from the NPS specimen. When restricted to the 29 PCR-detectable serotypes, Antonio et al. found that multiplex-PCR directly from the NPS-STGG specimen detected more serotypes per NPS than culture and sweep serotyping using the latex agglutination technique (153). PCR of the non-coding region of the pneumolysin (*ply*) gene followed by terminal-RFLP analysis improved detection of pneumococci and multiple serotype co-colonisation directly from NPS specimens in a Swiss study: this method could detect multiple serotypes down to a ratio of 1:100 (154). A pneumococcal microarray developed by the Bacterial Microarray Group (BμG@S) at St George's London correctly identified serotypes at a relative abundance of 1% in mixed NPS specimens or cultures (29).

1.8.2. Rationale for study of multiple serotype colonisation

Understanding the prevalence and dynamics of carriage of multiple pneumococcal serotypes is of considerable importance now that there is widespread deployment of pneumococcal conjugate vaccine and reports of increasing numbers of cases of invasive pneumococcal disease due to non-vaccine types (69). Immunisation with a vaccine covering only a few of the >90 serotypes may result in replacement disease by less commonly carried pneumococcal types (155). Pneumococcal serotypes compete within the nasopharynx, as evidenced by the increased colonisation by non-vaccine serotypes following introduction of PCV (156). This intra-species competition may be mediated by bacteriocins (157) or antimicrobial peptides of the innate immune system (158). In a murine model, resident pneumococci could prevent establishment of a new serotype introduced into the nasopharynx. This could be overcome by introducing a larger inoculum of the challenge serotype without affecting the growth of the resident serotype (159). However, in a rat model, pneumococcal strains of two serotypes 4 and 6B were able to co-exist in the nasopharynx, with the invading serotype becoming 25 – 90% of the total pneumococcal population over 96 hours (160). Competition between serotypes in the human nasopharynx has been mathematically modelled using Markov models and data

from longitudinal carriage studies performed in the UK and Kenya. In the UK, Melegaro and colleagues studied five serotypes (6A, 6B, 14, 19F, and 23F) and determined the "challenge strength" and "resistance" for each. Both of these competition parameters were lower for serotypes 19F and 23F than for 6A and 6B (161). Using the Kenyan dataset, Lipsitch et al. studied 27 serotypes and determined that serotype 19F was the serotype most resistant to competition (i.e. its presence in the nasopharynx resulted in lower rates of acquisition of other serotypes compared with non-colonised individuals) (162). Molecular approaches may clarify some of the contradictions noted when analysing datasets by serotype.

1.9. Transmission of pneumococci within the household

In the 1930-40s, it was demonstrated that family members of pneumococcal pneumonia patients were often colonised by the same pneumococcal serotype (144, 163). The detailed longitudinal studies on respiratory infections and upper respiratory tract colonisation performed in Paddington families in the 1950s identified that school aged children were most often associated with introduction and spread of pneumococci within the household (164, 165). Transmission occurred more frequently in crowded dwellings. The proportion of individuals in whom pneumococci could be detected increased in the days following the onset of coryzal symptoms, suggesting coryza enhanced pneumococcal transmission. Interestingly, studies of a cohort of 38 families in Syracuse, New York state did not find an association between crowding and transmission of pneumococci (166, 167). Hendley et al. determined that young children were more commonly colonised than older children or adults in a study of Virginian families (168). They found higher carriage rates in adults from households including young children compared with adults from households with older children or no children. Transmission within the family was common and often associated with upper respiratory tract infection (84). As part of the FinOM infant cohort, 100 families were also swabbed at the same time points as the study infant. After the age of

six months, carriage of the same pneumococcal type in another family member was the strongest predictor of carriage in the study infant (169). One hundred and twenty one complete UK families were swabbed monthly for 10 months in 2001-2. Young children were found to be important introducers of pneumococci into the household. Carriage in older family members was strongly associated with carriage in other household members (OR for carriage in the ≥5y age group if another family member colonised: 2.25 (95% CI 1.65 – 3.08)) (116). Subsequent modelling of this dataset confirmed that children are more likely to acquire pneumococci from the community than adults. Adults were found to be more infectious but less susceptible to infection than children. Transmission within the family was associated with larger households, i.e. the proportion of household-acquired colonisations increased as the number of people in the household increased (170). Pneumococcal colonisation and transmission has also been documented to be frequent in other crowded settings, such as day care centres (171, 172), orphanages (173), schools (174), and prisons (175).

Data regarding transmission of pneumococci in developing country settings are scarce. Darboe and colleagues modelled transmission of pneumococci within 196 Gambian mother-infant pairs. They concluded that there was a nine-fold increase in the odds of identifying a particular serotype in an infant if the same serotype was carried by the mother. However, they determined that only 9.5% of infant carriage could be attributed to carriage in the mothers (108). Nineteen complete Gambian households were studied bi-weekly for a year by Hill and colleagues. Their findings were in agreement with the UK study, in that individuals were more likely to be colonised if there were other colonised household members. A detailed molecular study of serotype 6B confirmed that children tended to initiate transmission of the strain within a household (79). Clustering of pneumococcal genotypes within households was also documented in a study of a Brazilian urban slum community (176).

1.10. Immune responses to pneumococcal colonisation

The immune response to pneumococcal colonisation is complex, most likely involving both B-cell and T-cell pathways, and remains incompletely understood. Some experimental murine models have suggested a relatively limited role for antibody in the clearance of nasopharyngeal colonisation and that a CD-4^+ T-cell pathway mediated by IL-17A is the dominant mechanism involved in immunity to pneumococcal colonisation (177, 178). In support of this are the results of a recent study which found greater IL-17A responses to pneumococcal antigens in adults and children from Bangladesh compared with those from Sweden, perhaps reflecting their greater exposure to pneumococci (179).

Using a mathematical modelling approach, Cobey and Lipsitch have demonstrated that a combination of weak serotype-specific immune responses (anti-capsular antibody mediated) and non-specific immunity (e.g. IL-17A or anti-protein antibody mediated) permit the on-going coexistence of >90 pneumococcal serotypes (180). The serotype-specific antibody responses act to stabilise competition, and non-specific immunity reduces fitness differences, between the serotypes.

1.10.1. Anti-capsular polysaccharide antibodies

Anti-capsular antibodies are an important defence against pneumococcal infection, and the capsular polysaccharides of 7 – 23 pneumococcal serotypes are included in current pneumococcal vaccines (3-6, 181, 182).

Colonisation in adult humans does result in a rise in serum serotype-specific anti-capsular polysaccharide immunoglobulin G (IgG) (183, 184), although these antibodies may not protect against nasopharyngeal acquisition (185). Nasopharyngeal exposure to pneumococci, in the absence of subsequent colonisation, did not induce a serum serotype-specific IgG response in an experimental colonisation model in adult humans (186). Pneumococcal conjugate vaccine studies in young children have shown that serum anti-capsular IgG protects against nasopharyngeal acquisition by, and may reduce colonisation

density of, pneumococcal serotypes covered by the vaccine (67, 156). Dagan and co-workers observed reduced nasopharyngeal acquisition of serotypes 14 and 19F in Israeli toddlers aged 12 – 35 months following administration of a nine-valent PCV: the probability of acquisition was inversely correlated with serum anti-capsular IgG concentration (187). In the control group of this study, previous nasopharyngeal exposure to a serotype protected against subsequent colonisation by the homologous serotype and, for serotypes 14 and 23F (but not the other serotypes studied), this was related to the production of specific anti-capsular serum IgG (120). In children aged less than two years, infant cohort studies and PCV vaccine studies have demonstrated the generally limited development of serotype-specific serum anti-capsular antibodies in the absence of PCV immunisation (67, 182, 188-192). However, these previous studies of pneumococcal colonisation and immune responses in the under two year-olds included relatively infrequent sampling points which restricted potential for the study of immune responses to individual pneumococcal serotype acquisitions.

1.10.2. Anti-protein antibodies

The next generation of pneumococcal vaccines would ideally protect against all serotypes by targeting conserved cell surface or virulence proteins, to avoid that issue of serotype replacement disease observed following the introduction of PCV (69, 193). Vaccines to several protein antigens have been demonstrated to provide protection against invasive pneumococcal disease in mouse models. These proteins include the pneumococcal pilus (PI-1, comprising three subunits RrgA, RrgB, and RrgC), pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumococcal surface protein C / choline binding protein A (PspC/CbpA), pneumolysin (Ply), histidine triad proteins (PhtA-E), neuraminidase (NanA), iron transport proteins (PiuA, PiaA) and also novel proteins, such as a protein required for cell wall separation of group B streptococcus (PcsB) and serine/threonine protein kinase (StkP), discovered by ANTIGENome scanning

(194, 195). Many of these proteins are important for successful pneumococcal colonisation and/or disease (196).

Anti-protein antibody development in response to pneumococcal colonisation has been studied in by several groups in a variety of settings. Whilst many of these proteins are well-conserved, some are not found in all pneumococci. This factor, coupled with the difference in immunogenicity between the various proteins, may explain some of the variation in antibody response seen (8, 197).

1.10.2.1. Finland infant cohort

Serum IgG concentrations to a variety of pneumococcal proteins were studied in up to 329 infants and their mothers. In this cohort, 87% of infants had been colonised by pneumococci by 24 months.

Development of antibodies to Ply, PsaA, and PspA was correlated with pneumococcal exposure. At 24 months the infant antibody geometric mean concentration (GMC) was greater than the mother GMC for anti-PsaA, similar to the mother GMC for anti-Ply, and less than the mother GMC for anti-PspA IgG (198). Most infants developed higher concentrations of IgG to the PspA family (family-1 or -2) they were exposed to but mothers had high serum concentrations of IgG to both PspA families (199). A higher serum anti-PspA IgG concentration at each time point predicted a greater risk of colonisation in the subsequent six months (200).

Serum IgG concentrations to Eno (α-enolase), Iga (immunoglobulin A1 protease), SlrA (streptococcal lipoprotein rotamase A), and PpmA (putative proteinase maturation protein A) were measured in infants with AOM. There were no differences in GMC when stratified by previous pneumococcal exposure, suggesting that the stimulus for antibody production was probably exposure to cross-reactive epitopes on other nasopharyngeal commensal organisms (201). Higher anti-PpmA antibody titres at 18 months of age reduced the risk of AOM in the subsequent six months (202).

Exposure to pneumococci resulted in higher serum IgG concentrations to PhtB, PhtE and CbpA, but at 24 months infant GMCs were lower than mother GMCs. For anti-PhtB and anti-PhtE, higher IgG concentrations were associated with increased risk of pneumococcal colonisation in the subsequent six months but there was no association with AOM risk (203). Higher anti-CbpA titres were associated with decreased risk of AOM in the subsequent 6 months (204).

Infant serum IgG concentrations to NanA were similar to adult levels by 24 months. There was no association found between anti-NanA antibodies and risk of subsequent pneumococcal colonisation or AOM (205).

1.10.2.2. Netherlands infant cohorts

The Netherlands Generation R study included a longitudinal analysis of antibody responses to 17 pneumococcal proteins, measured by Luminex assay, and pneumococcal carriage over the first two years of life in 57 infants (206). Four paired serum and NPS samples were collected between birth and 24 months of age. 17.5% of infants were colonised by 1.5 months of age. There was no evidence of protection against colonisation by maternal serum IgG to any of the proteins. Higher titres of antibodies to BVH-3, NanA, PpmA, PsaA, SlrA, SP0189, and SP1003 were associated with a decreased number of respiratory infections in the third year of life but none were associated with a reduction in pneumococcal colonisation. Another study from The Netherlands looked at colonisation and serum IgG to 18 pneumococcal proteins in infants followed from birth until 24 months of age as part of a PCV trial. Sera collected from infants at 12 and 24 months of age were analysed. Previous pneumococcal colonisation was associated with higher serum IgG titres but there was no evidence of protection from subsequent colonisation (207).

1.10.2.3. UK children

The relationship between pneumococcal carriage and antibodies to CbpA, Ply, PsaA, and PspA was assessed in a cross-sectional study of healthy children and those

undergoing adenoidectomy (aged 2 – 12 years). Serum IgG antibody concentrations to all but PsaA increased with age: anti-PsaA IgG concentration was static after five months of age. Serum IgG concentrations were higher in children non-colonised compared with pneumococcus colonised children over the age of two years, suggesting that pre-existing antibodies may have a protective effect against colonisation (208). Although these results agree with a similar study in younger Gambian children (209), the cross-sectional nature of the study invites caution when interpreting this finding: higher antibody levels in non-colonised children might reflect recent immune mediated clearance of colonisation and not protection per se. In the Finland studies described above, higher antibody levels at 24 months were generally positively correlated with pneumococcal exposure over the study period (198, 204).

1.10.2.4. Papua New Guinea infant cohort

Eighty nine mother-infant pairs were studied in the first four weeks of life to determine the relationship between mother and cord serum anti-Ply and anti-PspA (families 1 and 2) IgG titres on the timing of infant pneumococcal acquisition in an area where all infants are colonised by pneumococcus at a very early age (71). Three quarters (76%) of infants were colonised within four weeks of birth (median age 19 days). Maternal carriage at birth (30%, commoner in younger mothers) was associated with earlier infant colonisation and there was a trend to later colonisation in infants of older mothers. There was good correlation between mother and cord IgG titres, but cord titres were lower (median 7.2-fold for anti-Ply; 1.8-fold of anti-PspA family 1; 1.7-fold for anti-PspA family 2). There was good correlation between IgG titres to PspA family 1 and PspA family 2 titres, but not between anti-PspA1/2 and anti-Ply. There was no association between maternal antibody titres and carriage status at birth. In a multivariate model including all antibodies, and adjusting for maternal age and delivery carriage status, a higher maternal/cord anti-Ply titre was associated with delayed acquisition in the infant and a

higher maternal/cord anti-PspA family 1 (but not anti-PspA family 2) titre was associated with earlier acquisition.

1.10.2.5. Philippines infant cohort

Antibodies to various pneumococcal proteins were studied as part of the MATER cohort. Infants were followed during the first year of life and 90% had been colonised by pneumococci by six months.

Mother and cord blood antibody GMCs were similar for all three proteins. Infant antibody GMCs to Ply and PspA increased from 18 – 20 weeks but were still lower than mother-cord levels at 48 weeks. Anti-PsaA development was different: there was a rapid increase in GMC from the first infant sampling point (seven weeks) and the infant 14 week GMC was already greater than the mother GMC. Infants exposed to pneumococci had higher GMCs than non-exposed infants and this was significant for anti-PsaA and anti-Ply, but not for anti-PspA. A higher seven week anti-Ply concentration (i.e. maternally-derived antibodies) resulted in decreased risk of pneumococcal acquisition in the first three months of life (111).

Development of serum IgG antibodies to PhtD, CbpA, and LytC (an autolysin) in infants was correlated with pneumococcal colonisation. Anti-LytC antibody development was similar to that seen for antibodies to NanA (Finnish cohort) and PsaA (Philippines cohort): a more rapid increase in GMC in early infancy, reaching adults levels within the first two years of life. (210).

1.10.2.6. Gambian infant study

Forty Gambian infants were sampled between three and five months of age. The serum IgG anti-PsaA GMC was significantly lower in pneumococcal carriers than in non-carriers, suggesting a protective effect of anti-PsaA antibodies on carriage acquisition in early infancy. (209).

1.10.2.7. Kenyan cross-sectional study

A cross-sectional population survey of anti-PsaA, -PspA, and –Ply IgG was done in Kilifi. Serum anti-PspA and -Ply IgG concentrations increased in infancy and then levelled out with no decline observed in the elderly. Concentrations of serum anti-PsaA IgG were level throughout, indicating very early development in infancy (211).

1.10.2.8. Experimental human carriage studies

McCool and co-workers found that serum IgG to the N-terminal region of PspA prevented colonisation in adult humans and this molecule was immunogenic in those who became colonised (212). Analysis of serum IgG development in response to colonisation by 6B or 23F in human adults found that colonisation induced a significant serum IgG response to CbpA and PspA, but not to other proteins (PsaA, PpmA, Ply, IgA1 protease, and lipotechoic acid) (213).

1.11. Potential modifiers of pneumococcal colonisation dynamics

1.11.1. Pneumococcal pilus

Various cell-surface components, including pneumococcal surface adhesin A (PsaA), choline-binding protein A (CbpA), pneumococcal serine-rich repeat protein (PsrP) and pneumococcal adherence-virulence factor A (PavA) have been demonstrated to contribute to nasopharyngeal adherence and colonisation, but their mechanisms of action remain incompletely understood (46, 214-217). The RrgA subunit of the surface exposed pilus-1 filamentous structure also enhances pneumococcal adherence to respiratory epithelial cells *in vitro* (218, 219). Pneumococcal pilus-1 is encoded by the pilus islet-1 (PI-1; *rlrA* islet) and is composed of three subunits, RrgA, RrgB, and RrgC (219-221). Since immunisation with pilus antigens is protective against lethal intraperitoneal challenge in a mouse model, pilus subunits are regarded as potential candidates for inclusion in a protein-based pneumococcal vaccine (222). However, PI-1 presence is not universal: studies of

predominantly invasive pneumococci have found that PI-1 is present in isolates from a limited number of serotypes (particularly those included in the 7-valent conjugate vaccine, PCV7) and that its presence correlates with genotype by MLST (8, 197, 223-226). Given the prevalence of asymptomatic colonisation, and that serotype/genotype structure of invasive pneumococci is not stable (58), further work to describe the prevalence and function of the pilus-1 in pneumococcal carriage strains is warranted.

1.11.2. Bacterial and viral nasopharyngeal colonisers

1.11.2.1. Bacteria

Bacterial colonisation of the nasopharynx is a dynamic and interactive process. The nasopharynx is colonised with non-pathogenic (predominantly α-haemolytic streptococci) and potentially pathogenic micro-organisms (*Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus,* and *S. pneumoniae*) (227). Non-pathogenic members of the upper respiratory tract flora may inhibit the growth of potential pathogens (228). Johanson et al. found that 82% of oropharyngeal cultures from 101 healthy adults contained organisms, predominantly α-haemolytic streptococci, capable of inhibiting *S. pneumoniae* (229). Brook and Gober showed that a wide range of organisms could inhibit common respiratory pathogens. These "protective" organisms were found significantly less frequently in the nasopharynges of otitis media (OM) prone children compared with healthy controls (230). Faden et al. discovered that carriage of *S. pneumoniae, H. influenzae,* and *M. catarrhalis* increased significantly during episodes of otitis media, whilst the carriage of non-pathogens decreased (231). Pneumococcal vaccination has been associated with an increased incidence of acute otitis media caused by *S. aureus* (232). Several studies of children, from geographically diverse locations including The Gambia, Israel, The Netherlands and Papua New Guinea, have demonstrated a negative correlation between colonisation with *S. aureus* and *S. pneumoniae,* particularly those serotypes of pneumococcus covered by the seven valent conjugate vaccine (48, 233-235). Hydrogen

peroxide produced by pneumococcal strains may be responsible for this interference (236, 237), although a recent study has also implicated the pneumococcal pilus as a mechanism by which pneumococci inhibit co-colonisation with *S. aureus* (238). Interestingly, there was absence of a negative association between colonisation by *S. pneumoniae* and *S. aureus* in HIV positive South African infants suggesting the mucosal immune system may be important in the regulation of co-colonisation by these species (239). However, development of serum antibodies to *S. aureus* or *S. pneumoniae* does not appear to mediate this phenomenon (240). Previous studies have generally documented positive correlations between nasopharyngeal colonisation by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* (105, 235, 241-243). In a rat model of colonisation, Margolis et al. determined that colonisation by either *S. aureus* or *S. pneumoniae* enhanced subsequent colonisation by *H. influenzae*. Multiple strains of *S. aureus* were not able to co-exist: the resident strain was able to resist invasion by a newly introduced strain. Multiple strains of *S. pneumoniae* or *H. influenzae* were able to co-exist in the nasopharynx, although occasionally an immune response to *H. influenzae* colonisation limited nasopharyngeal acquisition of *S. pneumoniae* (160). Lysenko and colleagues also found that colonisation by *H. influenzae* could enhance clearance of *S. pneumoniae* from the nasopharynx by complement-dependent neutrophil killing (244). Increasing use of metagenomic sequencing techniques directly on nasopharyngeal specimens is likely to lead to a greater understanding of between-species colonisation dynamics (245, 246).

1.11.2.2. Viruses

The important role of viral infections as precursors of pneumococcal disease is well recognised. Infection by influenza viruses, respiratory syncytial virus, and rhinoviruses has been temporally linked to pneumococcal disease in both historical and contemporary studies (247-253).

Co-infection with bacteria is common in "viral" ARI, occurring in 3 – 30% of cases (254). Madhi et al. recently reported the results of a PCV9 vaccine study involving nearly 40,000 South African infants (255). They demonstrated that, in addition to a 20% reduction in total number of episodes of pneumonia, a 31% reduction in the number of cases of virus-associated pneumonia was seen in infants receiving the vaccine compared to the control group. Pneumonias associated with RSV decreased by 22% and those associated with influenza A decreased by 45%, suggesting that *S. pneumoniae* super-infection occurs in a significant proportion of virus-associated pneumonias.

Viral infection of the respiratory tract predisposes to bacterial colonisation/super-infection by several mechanisms (256, 257). Non-specific mechanisms include viral-induced damage to the respiratory epithelium, resulting in impairment of ciliary function. Influenza neuraminidase may expose pneumococcal receptors on the upper respiratory tract epithelium, thus increasing adherence of *S. pneumoniae* (258). Also, in a murine model, influenza A infection increases type 1 interferon production resulting in decreased macrophage-mediated clearance of colonising pneumococci from the nasopharynx (259). Virus-mediated up-regulation of the platelet activating factor receptor may also enhance adhesion of pneumococci to the respiratory epithelium (260). Similarly, RSV infection increases pneumococcal adherence to the upper respiratory tract epithelium and this is mediated by the RSV G glycoprotein (261, 262).

1.12. Study rationale and objectives

1.12.1. Rationale for the study

As stated previously, *S. pneumoniae* is estimated to be responsible for ~10% of deaths in children aged <5 years (2). Since almost a third of the world's children live in Southeast Asia, this pathogen is likely to be of considerable importance but there is a paucity of data on pneumococcal colonisation and disease in the region (2, 63, 65).

Infant immunisation with pneumococcal conjugate vaccines is now routine in many countries including those that are GAVI eligible (263). In addition to the prevention of invasive disease, pneumonia, otitis media and all-cause mortality, PCVs have been shown to reduce colonisation by the serotypes covered by the vaccine which has led to adaptive alterations in the serotypes colonising the nasopharynx and causing disease (67, 68). Surveillance of colonisation is an important component of the vaccination monitoring process and robust pre-vaccine era data are important in the planning of introduction and assessment of impact (69).

Given the large number of serotypes and many potential modifying agents, including other nasopharyngeal colonisers, respiratory virus infection, infant immune system maturation and antibiotic use, pneumococcal colonisation dynamics are best understood from longitudinal data (47, 72, 73, 76, 89, 105-110, 112-116). Such studies are difficult to perform so few have been comprehensive with regard to follow-up duration, consistency of sampling intervals, and clinical data collection to permit assessment of potential modifiers of pneumococcal carriage. Various sampling and culture methods have been described, adding to the heterogeneity between studies and prompting the development of a WHO standard protocol for pneumococcal nasopharyngeal colonisation detection (70).

1.12.2. Research question

What is the natural history and outcome of *Streptococcus pneumoniae* colonisation in infancy in the presence or absence of specific maternal and infant immunity and simultaneous colonisation/infection with other bacteria and viruses?

1.12.3. Hypotheses to be tested

- Early nasopharyngeal colonisation with *S. pneumoniae* leads either to an immune response to the bacterium, with subsequent protection from disease and eradication, or to carriage, or to an episode of clinical disease.

- Current methods of estimating nasopharyngeal pneumococcal carriage lack sensitivity, and improved methods, particularly focussed on identifying simultaneous multiple carriage, would significantly add to our understanding of nasopharyngeal carriage acquisition, duration and disease.
- Pneumococcal acquisition can be profoundly influenced by simultaneous viral infection or colonisation of the nasopharynx.

1.12.4. Additional aims and objectives

- Determine the frequency of transmission of pneumococcal strains between mother and infant.
- Assess the contribution of maternally-derived antibodies to the timing of first pneumococcal colonisation in infants.

2 Methods

2.1. Study site

Maela is a camp for displaced persons from Myanmar (Burma), located approximately 500 km from Bangkok in Tak province on the North-western border of Thailand (Figure 2). The camp covers an area of 4 km^2 and is situated in forested hills approximately five kilometres from the border with Myanmar (Figure 3 & Figure 4). The climate is tropical, with three defined seasons: hot (March – May), wet (June – October), and cool (November – February) (Figure 5).

Figure 2. Map of Thailand and Myanmar

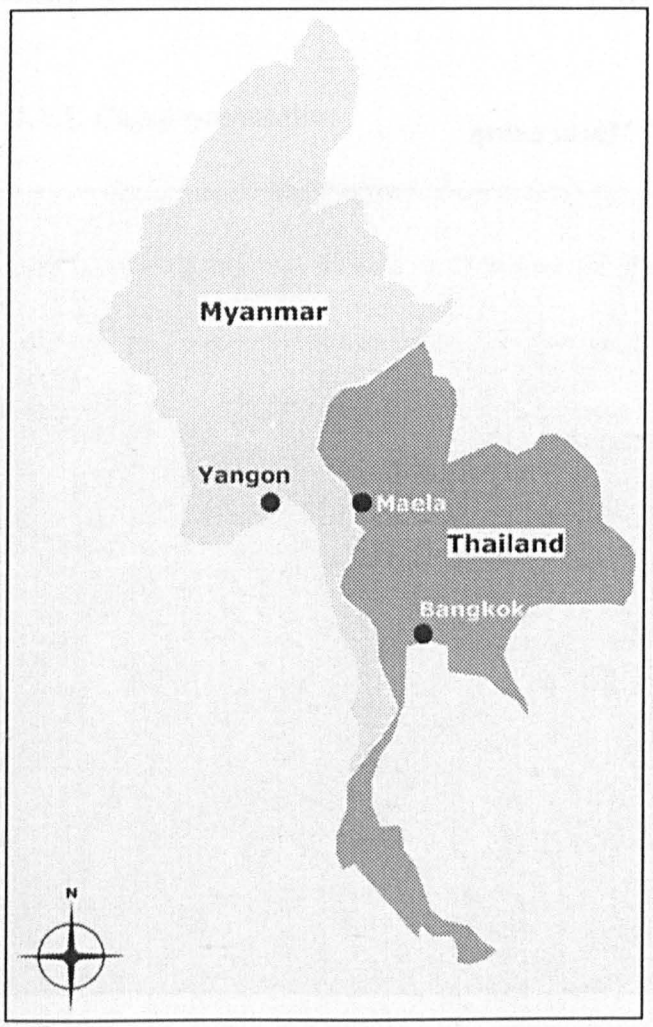


Figure 3. Satellite image of Maela camp

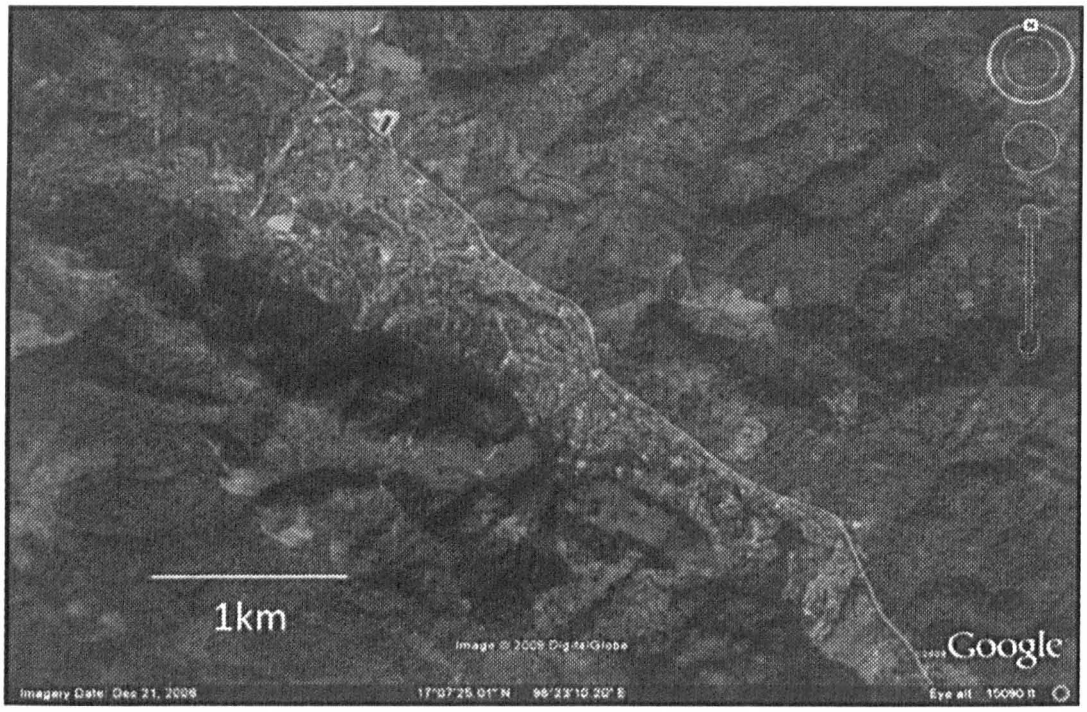


Figure 4. Photograph of Maela camp

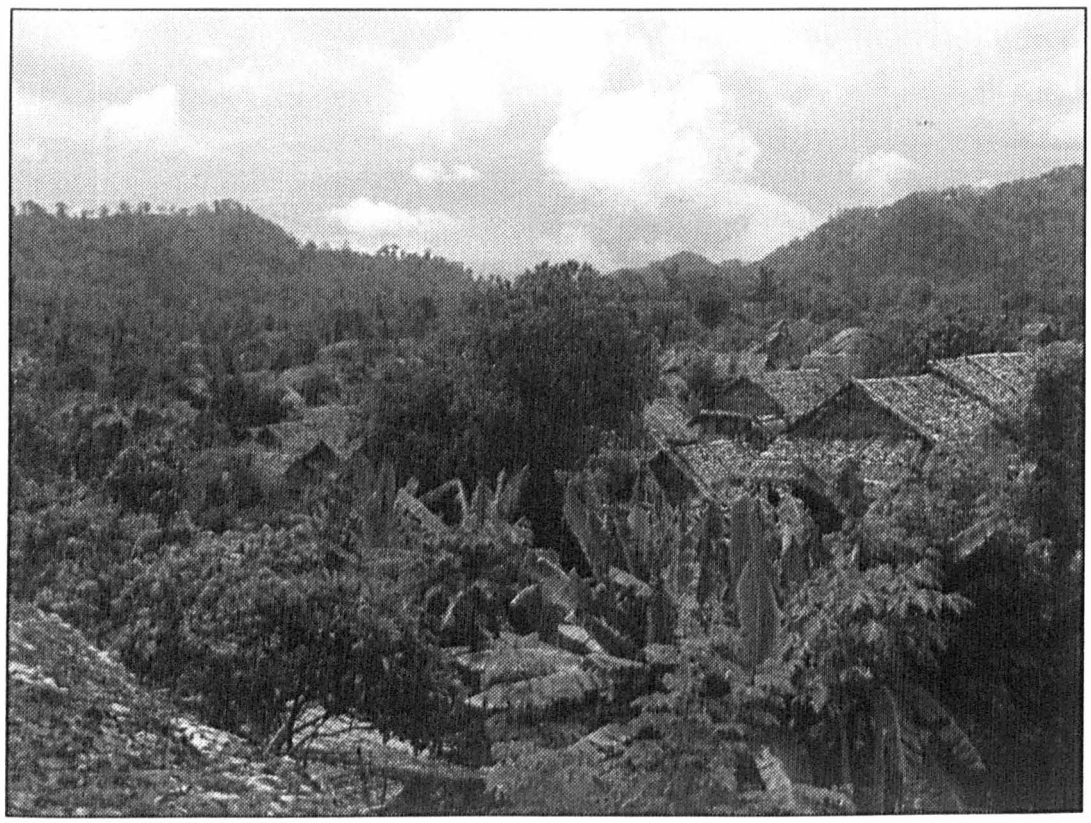
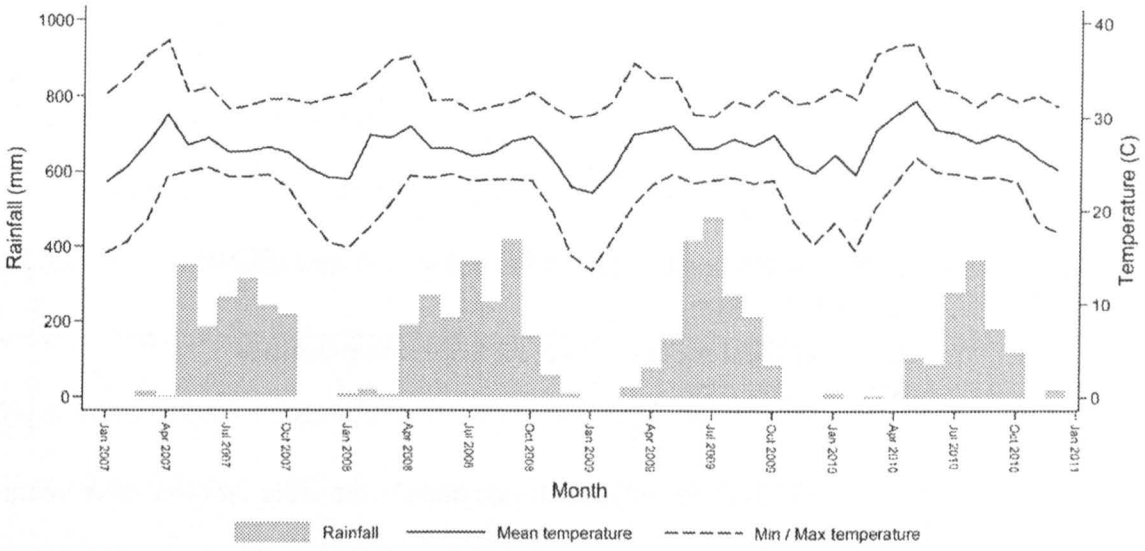


Figure 5. Meteorological data for Mae Sot, 2007 – 2010

(Data provided by Mae Sot Meteorological Station, Thai Meteorological Department)



2.1.1. Camp population

Refugees from Myanmar began arriving at Maela in 1984. In September 2007 the official population was 42,652, accounting for 30.1% of all Burmese officially registered refugees in Thailand (figures from http://www.tbbc.org/camps/populations.htm). The Karen are the predominant ethnic group living in Maela.

2.1.2. Health in Maela

At the commencement of the study, only aggregate health statistics were available for the refugee population on the Thailand-Myanmar border: camp-specific data were not published in the annual CCSDPT (Committee for Coordination of Services to Displaced Persons in Thailand) report (264). Comparing these data with WHO figures for Myanmar and Thailand demonstrates that neonatal, infant, and <5 year mortality figures fell between those for Myanmar and Thailand (Table 5) (265).

Table 5. Key health statistics for border refugee camps, Myanmar, and Thailand

Health indicator	Myanmar (265)	Thailand (265)	Border camps (264)
Infant Mortality Rate (/1000 live births)	50 (2010)	11 (2010)	20 (2006)
Neonatal Mortality Rate (/1000 live births)	32 (2010)	8 (2010)	11 (2006)
<5yr Mortality Rate (/1000 population)	66 (2010)	13 (2010)	28 (2006)
Deaths <5 due to pneumonia (%) (266)	19 (2004)	11 (2004)	9[a] (2006)

[a] Reported as deaths due to LRTI and not specifically pneumonia

In 2006, 15% of the refugee population were less than five years of age and only 9% were 45 years or older. Lower respiratory tract infections (LRTI) were the cause of 9% of deaths, and were responsible for 25% of all reported morbidity, in the under-5 age group (264).

General healthcare is provided to the camp population by the NGO Premiere Urgence-Aide Médicale Internationale (PU-AMI), who have both in-patient and out-patient facilities staffed by a combination of local medical staff and expatriate physicians. PU-AMI is also responsible for public health activities, including infant immunisations. WHO Expanded Program on Immunisation (EPI) recommended immunisations are offered to camp residents, and in 2006 CCSDPT reported >90% coverage for all infant immunisations in the refugee population (264). Pneumococcal conjugate, Hib, and influenza vaccines are not available in the camp.

Antenatal care and malaria treatment has been provided by Shoklo Malaria Research Unit (SMRU) since 1986. Approximately one hundred locally trained medics, nurses, and home visitors run the SMRU clinic in Maela, under the supervision of expatriate physicians.

2.2. Study design

The work contained in this thesis formed part of a larger cohort study of infant pneumonia epidemiology carried out at Maela between 2007 and 2010.

2.2.1. Sample size calculation

For the overall cohort, the sample size calculation was based on observation of clinical pneumonia episodes. Using the published estimated incidence of clinical pneumonia in developing world children (<5y) of 0.28 episodes/child-year (267), a sample size of 1,000 infants followed for two years would be expected to result in the observation of 560 (95% CI 520 – 600) pneumonia episodes. Assuming a 1% per month dropout rate, this would result in 886 infants being followed for 12 months and 786 being followed for the entire 24 months, potentially permitting the observation of approximately 520 clinical pneumonia episodes.

For the pneumococcal carriage study, a 1 in 4 randomisation at recruitment would result in 250 mother-infant pairs being included. Assuming an overall carriage prevalence of 80% in infants and a 1% per month dropout rate, approximately 4,500 pneumococci would be isolated. Assuming a carriage prevalence of 20% in the mother, approximately 1,100 pneumococci would be isolated. These figures would permit detection of serotypes present at 1% of the total carried pneumococcal population. Given that its prevalence is likely to be underestimated by standard culture methodology, the detection of multiple pneumococcal serotype colonisation was not included as a factor in the sample size calculation (70, 145).

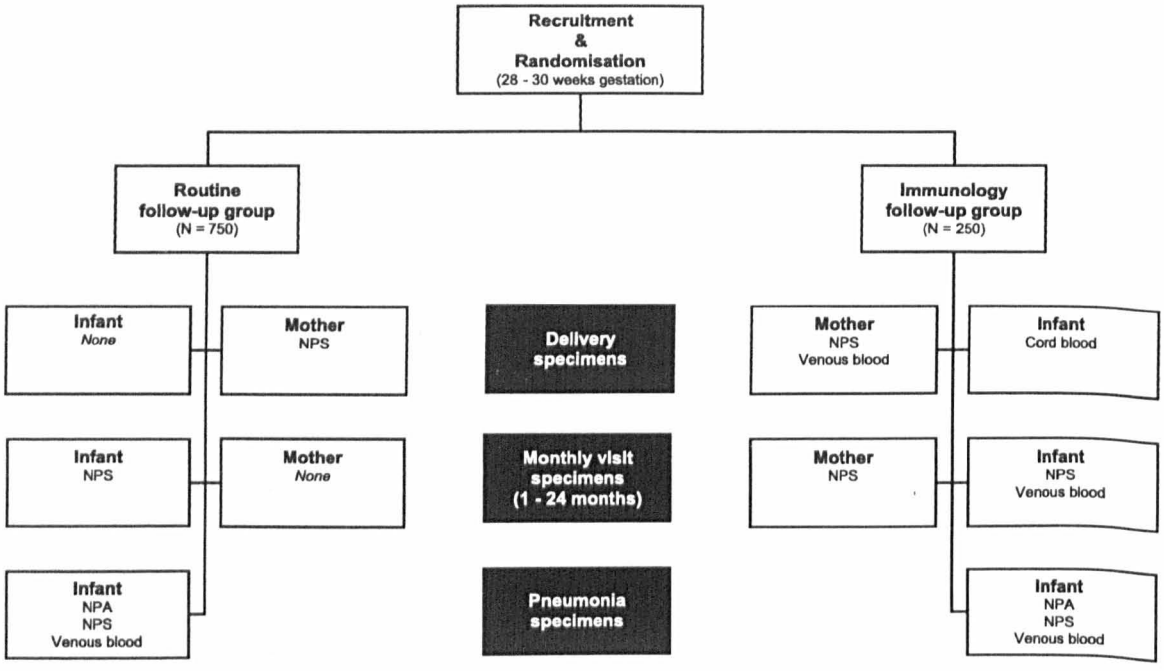
2.2.2. Recruitment

Between October 2007 and November 2008, all pregnant women attending the SMRU antenatal clinic at 28 – 30 weeks gestation were invited to consent to their infant's participation in the cohort study. One thousand allocation codes were generated by Dr Verena Carrara, SMRU epidemiologist: 750 for participation in the "routine" follow-up

group and 250 for participation in the "immunology" follow-up group. Using sealed opaque envelopes containing an allocation code, women were randomly allocated to either the "routine" or "immunology" follow-up groups (Figure 6).

The only exclusion criterion was related to likely departure from Maela during the study follow-up period: women who were actively planning resettlement to a third country were not eligible for study enrolment.

Figure 6. Cohort design and follow-up



Women were encouraged to deliver at the SMRU clinic and all were reviewed around the time of delivery. Mothers were asked to bring their infant to monthly follow-up visits until the infant reached 24 months of age. Specimen collection varied by follow-up group (Figure 6). Mothers were also requested to bring their infant for review at the SMRU clinic when unwell for whatever reason. All study activities occurring in Maela were supervised by Dr Claudia Turner, SMRU consultant paediatrician.

2.2.3. Study visits

2.2.3.1. Delivery visit

All women had a NPS collected around the time of delivery to determine pneumococcal colonisation status. Additional specimens were collected from mothers randomised to the "immunology" follow-up group: 5 mL venous blood was taken from both mother and umbilical cord. Details regarding the woman's health, pregnancy history, and household structure were recorded (Appendix 1). Following delivery, study infants underwent a full newborn examination (Appendix 1).

In the event of a home delivery, women were requested to attend the SMRU clinic with their infant for review as soon as possible after delivery.

2.2.3.2. Monthly visits

At each monthly visit, a health questionnaire was completed to document illnesses occurring in the previous month. Anthropometric measurements, including weight, height, and arm circumference, were taken at these visits (Appendix 1). A nasopharyngeal swab (NPS) was taken from all infants. Mothers in the "immunology" follow-up group also had a NPS taken. Immunology follow-up group infants had a 0.5 mL venous blood specimen collected at each visit, with larger volumes of blood (5 mL) being collected at the 6, 12, 18, and 24 month visit.

In the event of a missed monthly visit, home visitors made contact with the family at home to reschedule the appointment.

2.2.3.3. Illness visits

Infants were reviewed at the SMRU clinic during illness episodes. Clinical pneumonia was diagnosed following the WHO Integrated Management of Childhood Illnesses (IMCI) criteria (Table 6) (268) , and included the "very severe" pneumonia

category as specified in the WHO Pocket Book of Hospital Care for Children (269). Clinical data were recorded on a standard illness CRF (Appendix 1).

All children diagnosed with pneumonia had a chest x-ray (Mae Ramat hospital, Mae Ramat, Thailand), nasopharyngeal aspirate (NPA), NPS, and venous blood taken. Chest x-rays were interpreted in accordance with the WHO standardised criteria (270). Treatment followed SMRU Paediatric Guidelines.

Table 6. Clinical pneumonia diagnostic criteria

Pneumonia severity	Definition	Notes
Pneumonia	Cough *OR* difficulty breathing *AND* Fast respiratory rate[a]	Fast RR definition: <2 months: ≥60 2 – 11 months: ≥50 12 – 59 months: ≥40
Severe pneumonia	Cough *OR* difficulty breathing *AND* Lower chest wall indrawing	Fast RR may not be present
Very severe pneumonia	Cough *OR* difficulty breathing *AND* Central cyanosis *OR* severe respiratory distress *OR* inability to drink	Fast RR may not be present

[a] Measured over one minute

2.2.4. Specimen collection

2.2.4.1. Nasopharyngeal swabs

Nasopharyngeal swabs were collected in accordance with the WHO standard protocol for detection of pneumococcal nasopharyngeal colonisation (70). A Dacron-tipped flexible shaft swab (MW151D; Medical Wire & Equipment, Corsham, UK) was inserted into the nasopharynx, rotated, and withdrawn. If significant resistance was encountered the other nostril was used. The swab tip was excised immediately into a cryovial containing 1 mL STGG medium (skimmed milk, tryptone, glucose, glycerol medium; prepared in-

house) using 70% ethanol-cleaned scissors. NPS-STGG specimens were transferred to the SMRU laboratory in Mae Sot in a cool box, within eight hours of collection, and frozen at -80°C until culture. All specimens were vortexed for 15 seconds prior to freezing.

2.2.4.2. Nasopharyngeal aspirates

A sterile 8-French infant feeding tube was inserted into the nasopharynx and then withdrawn while suction was applied with a 20 mL syringe attached to the feeding tube. The nasopharyngeal secretions and the tip of the feeding tube were transferred to a cryovial containing 1 mL viral transport medium (VTM, MEM-Hanks' with 0.5% gelatin, amphotericin B, penicillin, streptomycin; prepared in-house) and were transferred daily to the Mae Sot laboratory in a cool box and frozen at -80 °C until analysis by rRT-PCR.

2.2.4.3. Serum specimens

Venous blood was collected into plain blood tubes (Teklab, Sakriston, UK). Serum was separated by centrifugation at 3,000 rpm for 10 minutes in the Maela clinic laboratory. Serum specimens were transferred daily to the Mae Sot laboratory in a cool box and frozen at -80 °C until analysis.

2.2.5. Specimen processing

All core bacteriology and molecular work was done in the SMRU microbiology laboratory (Mae Sot, Thailand). Additional work was performed in collaborating laboratories as described below and in the subsequent chapters.

2.2.5.1. Nasopharyngeal swabs

2.2.5.1.1. General comments

Nasopharyngeal swabs were cultured in batches of between 50 and 150 per week. Prior to culture, the NPS-STGG specimens were fully thawed and vortexed for 15 seconds.

2.2.5.1.2. Detection of *Streptococcus pneumoniae*

Using disposable calibrated loops (Sterilin, Newport, UK), 10 µL of thawed NPS-STGG was streaked onto 5% sheep blood-CNA agar (bioMerieux, Marcy L'Etoile, France) and incubated overnight at 36 °C in 5% CO_2. In the absence of growth, plates were incubated for a further 24 hours before being discarded. All morphologically distinct alpha-haemolytic colonies were sub-cultured onto plain 5% sheep blood agar (Clinical Diagnostics, Bangkok, Thailand). In the absence of morphologic variation, two representative colonies were sub-cultured. *S. pneumoniae* was identified by colonial morphology and optochin disc susceptibility (5 µg / 6 mm optochin disc; Oxoid, Basingstoke, UK), defined as zone diameter of ≥14 mm. Isolates with reduced optochin disc zone diameters (7 – 13 mm) were confirmed as pneumococci by the tube bile solubility test (271). Where colonial morphology and optochin zone diameter was identical in both sub-cultured colonies, only one of the isolates was followed further. Whilst this colony selection methodology might underestimate multiple serotype colonisation, it is likely to identify the dominant serotype in each specimen as well as correctly identify the most common serotypes in a study population (148, 272). The standardised WHO protocol permits direct comparison with other datasets using the same methodology.

2.2.5.1.3. Detection of other bacterial species

For infant specimens only, a further 10 µL of thawed NPS-STGG was streaked onto a chocolate agar plate (Clinical Diagnostics, Bangkok, Thailand), a 10 unit bacitracin disc (Oxoid, Basingstoke, UK) applied to the first streak, and the plate incubated overnight at 36 °C in 5% CO_2. In the absence of growth, plates were incubated for a further 24 hours before being discarded.

Target organisms were *Haemophilus influenzae, Moraxella catarrhalis,* and *Staphylococcus aureus*. These organisms were identified using standard microbiological techniques as outlined in Table 7.

Table 7. Identification of non-pneumococcal nasopharyngeal colonisers

Organism	Identification tests	Notes
Haemophilus influenzae	Bacitracin[a] resistant	Serotyping by slide agglutination[b]
	Gram stain	
	X+V[a] factor dependent growth	
Moraxella catarrhalis	Typical colony characteristics	
	Gram stain	
	Trybutyrin positive[c]	
Staphylococcus aureus	Gram stain	Growth on both CNA-blood and chocolate
	Catalase positive	plates
	Coagulase	*S. aureus*: coagulase and DNase positive
	DNase	CoNS: coagulase and DNase negative

[a] Oxoid, Basingstoke, UK
[b] Becton Dickinson, Franklin Lakes NJ, USA
[c] Rosco Diatabs, Taastrup, Denmark

2.2.5.2. Further work on *Streptococcus pneumoniae* isolates

2.2.5.2.1. Serotyping

All pneumococcal isolates were serotyped by latex agglutination (19, 273). Latex serotyping reagents were prepared in-house, using a protocol kindly supplied by Prof Richard Adegbola (MRC Laboratories, The Gambia). Briefly, a 0.5 McFarland suspension of pure overnight culture of pneumococci was made in 0.85% saline. This suspension was first tested with latex antisera pools A – I, followed by all appropriate group, type, and factor latex antisera. Ten microliters of pneumococcal suspension was mixed with the same volume of latex antiserum on a clean glass slide. The slide was rocked on an orbital shaker for up to two minutes. A positive result was indicated by clear agglutination with clearing of the background. Traditional serotyping, using the Quellung reaction, was performed on isolates with equivocal latex serotype results (274). Serotype 6A isolates were confirmed as either serotype 6A or 6C by PCR, as described elsewhere (275).

Non-typeable pneumococci, either morphologically typical pneumococcal colonies (likely encapsulated organisms) or rough colonies (likely non-encapsulated organisms)

were provisionally identified when weak agglutination with pool B and serogroup 19 latex antisera, but no agglutination with group 19 factor antisera (19b, 19c, 19f, 7h), was observed. These isolates, along with those completely non-reactive with typing antisera, were confirmed by bile solubility and absent capsular swelling with Omniserum (SSI Diagnostica, Statens Serum Institute, Hillerod, Denmark) (274). To avoid over-estimation of the prevalence of NT pneumococci as a result of capsule locus loss during prolonged or multiple sub-culture, serotyping was performed on colonies picked from overnight cultures only (123). If repeat serotyping was subsequently required, it was performed on colonies picked from overnight culture of isolates stored at -80 °C in STGG since primary isolation.

2.2.5.2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibilities were determined for all *S. pneumoniae* isolates. The modified Kirby-Bauer disk diffusion method was used and zone diameter were interpreted using CLSI guidelines (2007 version, Table 8) (276, 277). Minimum inhibitory concentrations for benzyl penicillin and ceftriaxone were determined by the Etest method (AB Biodisk, Solna, Sweden) for isolates with an oxacillin disc zone diameter of <20 mm (Table 9) (276). Etests were performed in accordance with manufacturer's instructions with the exception that organism suspensions were made in 0.85% saline solution rather than broth, following confirmation from AB Biodisk that this was an acceptable alternative.

Table 8. Zone diameter interpretive criteria for *Streptococcus pneumoniae*

Drug	Disc (μg)[a]	Zone diameter (mm)		
		Resistant	Intermediate	Susceptible
Oxacillin[b]	1	-	-	≥20
Erythromycin	15	≤15	16 - 20	≥21
Clindamycin	2	≤15	16 - 18	≥19
Tetracycline	30	≤18	19 - 22	≥23
Chloramphenicol	30	≤20	-	≥21
Co-trimoxazole	1.25/23.75	≤15	16 - 18	≥19

[a] Oxoid, Basingstoke, UK
[b] To determine penicillin susceptibility

Table 9. MIC interpretive criteria for *Streptococcus pneumoniae*

Drug	MIC (μg/mL)		
	Resistant	Intermediate	Susceptible
Benzyl penicillin	≥2	0.12 - 1	≤0.06
Ceftriaxone	≥4	2	≤1

2.2.5.3. Nasopharyngeal aspirates

NPA specimens were processed in weekly batches. Viral nucleic acid was extracted from the thawed NPA-VTM specimens using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracts were analysed by rRT-PCR for adenoviruses, human metapneumovirus, influenza viruses (influenza A (seasonal H1N1, seasonal H3N2 and pandemic H1N1) and influenza B), and respiratory syncytial virus, as described elsewhere (278, 279). A human RNaseP PCR was used to detect the presence of inhibitors in the NPA-VTM specimens (280). A Rotorgene 6000 real-time PCR thermocycler (Corbett Life Science, Sydney, Australia) and SuperScript III One-Step RT-PCR kits (Invitrogen, Carlsbad CA, USA) were used throughout. Positive and negative controls were included in every PCR run.

Specimens were considered positive if a virus PCR cT value was <40 with appropriate run control results. Specimens with low positive PCR results (cT values of 35

– 39) were repeated: only if the cT was <40 in both runs was the virus PCR considered positive.

2.2.5.4. Quality control in the SMRU microbiology laboratory

Written SOPs were developed for all laboratory activities (Appendix 2).

All new batches of culture media (commercial or in-house prepared) were tested for sterility, the ability to support growth of target organisms, and, where appropriate, the ability to inhibit non-target organisms. Antimicrobial discs were quality controlled on a weekly basis according to CLSI guidelines (276). Similarly, Etests were tested on a monthly basis. All bacterial identification tests were performed with appropriate positive and negative controls.

The molecular laboratory workflow was strictly controlled to avoid contamination: master mix preparation, nucleic acid extraction, amplification, and gel electrophoresis took place in separate laboratory rooms.

The laboratory participated in the Thailand national external quality assurance scheme (DMSc unit code MI 1158). In 2008, the laboratory passed a one-off US-CDC organised quality assurance exercise for respiratory virus detection.

2.2.5.5. Serum specimens

All serology work was performed by staff at the WHO reference laboratory for pneumococcal serology in the Institute of Child Health (London, UK), under the supervision of Prof David Goldblatt. This work is described in sections 5.1.4.3 and 5.2.4.3.

2.2.6. Data management and analysis

Clinical data were double-entered into an Access 2003 database (Microsoft, Richmond WA, USA) and the databases merged to identify data entry errors. Laboratory data were single-entered into the database and all entries were checked against the original

laboratory CRFs at the completion of the study. Statistical analyses were performed in Stata/IC 12.1 (StataCorp, College Station TX, USA).

2.2.7. Ethical considerations

Written informed consent was obtained from the women prior to study enrolment. Participant information sheets and consent forms were translated into both Karen and Burmese languages. In the event of a woman being unable to read or write, the information sheet was read out loud by one of the consent team, her thumbprint used in place of a written signature, and the consent form counter-signed by a witness.

Ethical approval was granted by the ethics committees of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM-2007-014 / MUTM-2009-306) and Oxford Tropical Research Ethics Committee, Oxford University, UK (OXTREC-031-06).

3 Pneumococcal colonisation dynamics in infants and mothers

3.1. A longitudinal study of *Streptococcus pneumoniae* carriage in a cohort of infants and their mothers on the Thailand-Myanmar border

3.1.1. Summary

Background

Pneumococcal disease is a major cause of childhood death. Almost a third of the world's children live in Southeast Asia, but there are few data from the region on pneumococcal colonisation or disease. The study aim was to document the characteristics of pneumococcal carriage in an isolated SE Asian birth cohort.

Methods

Two hundred and thirty four Karen mother-infant pairs were studied. Infants were followed from birth and nasopharyngeal swabs were taken from mother and infant at monthly intervals until the infant was 24 months old. NPS were cultured following WHO methodology and all cultured pneumococci were serotyped by latex agglutination and had antimicrobial susceptibilities determined by disc diffusion testing.

Results

8,386 swabs were cultured and 4,396 pneumococci characterised. Infants became colonised early (median 46 days; 95% CI 45 - 46) and by 24 months had a median of seven (range 0 - 15) carriage episodes. Maternal smoking and additional young children in the house were associated with earlier colonisation (HR 1.5 (95% CI 1.1 - 2.1) and 1.4 (95% CI 1.0 - 1.9)). For the four commonest serotypes and non-typeable pneumococci, previous exposure to homologous or heterologous serotypes resulted in an extended interval to reacquisition of the same serotype. Previous colonisation by serotypes 14 and 19F was also associated with reduced carriage duration if subsequently reacquired (HR for first reacquisition 4.1 (95% CI 1.4 - 12.6) and 2.6 (1.5 - 4.7)). Mothers acquired pneumococci less frequently, and carried them for shorter periods, than their infants (acquisition rate 0.5

vs. 1.1 /100 person-days, $P < 0.001$; median duration 31 vs. 61 days, $P = .001$). 55.8% of pneumococci from infants were vaccine serotypes (13-valent pneumococcal conjugate vaccine, PCV13), compared with 27.5% from mothers ($P < 0.001$). Non-typeable pneumococcal carriage was common, being carried at least once by 55.1% of infants and 32.0% of mothers. 34.6% and 26.9% of pneumococci from infants and mothers, respectively, were multi-drug resistant. Infants co-habiting with other young children were at greater risk of being colonised by drug resistant pneumococci (OR 1.2 (95% CI 1.2 – 1.8)).

Conclusions

Pneumococcal carriage frequency and duration are influenced by previous exposure to both homologous and heterologous serotypes. These data will inform vaccination strategies in this population.

3.1.2. Introduction

Streptococcus pneumoniae is estimated to be responsible for ~10% of deaths in children aged <5 years (2). Since almost a third of the world's children live in Southeast Asia, this pathogen is likely to be of considerable importance but there is a paucity of data on pneumococcal disease in the region (2, 63, 65). Infant immunisation with pneumococcal conjugate vaccines is now routine in many countries including those that are GAVI eligible (263). In addition to the prevention of invasive disease, pneumonia, otitis media and all-cause mortality, PCVs have been shown to reduce colonisation by the serotypes covered by the vaccine which has led to adaptive alterations in the serotypes colonising the nasopharynx and causing disease (67, 68). Surveillance of colonisation is an important component of the vaccination monitoring process and robust pre-vaccine era data are important in planning and assessment of impact (69). Given the large number of serotypes and many potential modifying agents, including other nasopharyngeal colonisers, respiratory virus infection, infant immune system maturation and antibiotic use,

pneumococcal colonisation dynamics are best understood from longitudinal data (47, 72, 73, 76, 89, 105-110, 112-116). Such studies are complicated to perform so few have been comprehensive with regard to follow-up duration, consistency of sampling intervals, and clinical data collection to permit assessment of potential modifiers of pneumococcal carriage. Various sampling and culture methods have been described, adding to the heterogeneity between studies and prompting the development of a World Health Organisation standard protocol for pneumococcal nasopharyngeal colonisation detection (70).

3.1.3. Aims and objectives

The objectives of this study were to explore pneumococcal colonisation dynamics and modifiers in mother-infant pairs followed from birth for 24 months. The study was nested within a longitudinal study designed to establish the epidemiology and aetiology of pneumonia in a cohort of refugee children aged <2 years. The behaviour of serotypes included in the current conjugate vaccines was of particular interest, as they are the most prevalent serotypes causing disease and conjugate vaccines are known to interfere with pneumococcal transmission by perturbing nasopharyngeal colonisation.

3.1.4. Methods

3.1.4.1. Study site and population

Between October 2007 and November 2008, all pregnant women attending the SMRU antenatal clinic in Maela camp were invited to consent to their infant's participation in the pneumonia cohort study. Using sealed opaque envelopes containing an allocation code, women were randomly allocated to the "immunology" (pneumococcal carriage) follow-up group at enrolment. Women enrolled into this "immunology" follow-up group had an NPS taken at delivery and both infant and mother had a NPS taken at monthly surveillance visits from 1 – 24 months of age.

3.1.4.2. Sampling and laboratory procedures

NPS were collected according to the WHO pneumococcal colonisation detection protocol and processed as described in sections 2.2.4.1 and 2.2.5.1 (70). Briefly, nasopharyngeal secretions were sampled using Dacron-tipped swabs (Medical Wire & Equipment, Corsham, UK) and the tip was immediately stored in a cryovial containing 1ml STGG medium. NPS-STGG specimens were transferred to the laboratory in a cool box within eight hours of collection and were frozen at -80 °C until culture. Ten microliters of thawed STGG was cultured onto sheep blood-CNA agar (bioMerieux, Marcy L'Etoile, France) and incubated overnight at 36 °C in 5% CO_2. All morphologically distinct alpha-haemolytic colonies were sub-cultured onto plain sheep blood agar. *S. pneumoniae* was identified by colonial morphology, optochin disc susceptibility +/- bile solubility testing (271). Pneumococci were serotyped by latex agglutination and confirmed by Quellung typing if equivocal (19, 273). Serotype 6C was identified by PCR (275). Non-typeable isolates were confirmed by bile solubility and absence of capsular swelling with Omniserum (SSI Diagnostica Statens Serum Institute, Hillerod, Denmark) (274). Antimicrobial susceptibilities were determined by disk diffusion, following 2007 CLSI guidelines (276, 277). Etests (AB Biodisk, Solna, Sweden) were used to determine MICs to benzyl penicillin and ceftriaxone for all isolates with a growth inhibition zone diameter of <20 mm around the 1 μg oxacillin disc.

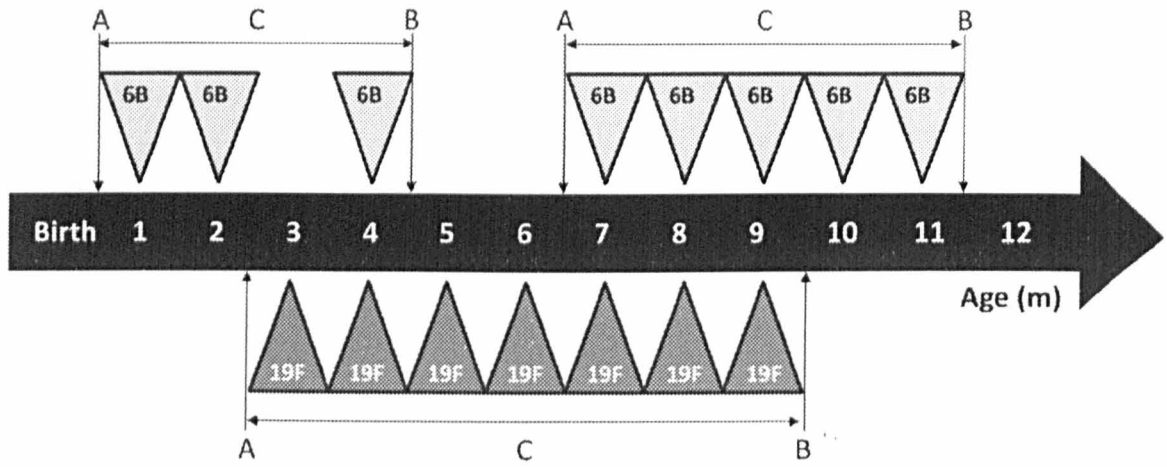
3.1.4.3. Definition of carriage

A carriage episode was defined as the period of time between acquisition and clearance of a pneumococcal serotype. Acquisition was identified when a pneumococcal serotype was cultured from a swab for the first time or when a serotype was re-cultured following clearance as defined below. Carriage episodes commenced at the midpoint between the last negative swab and the first positive swab for the serotype. Following acquisition, clearance of the serotype from the nasopharynx was considered to have

occurred when two consecutive swabs were culture negative for that serotype. Termination of the carriage episode was defined as the midpoint between the last positive swab and the first negative swab for the serotype (Figure 7) (73). Given the lability of their capsules, serotypes 15B and 15C were considered as a single serotype, labelled 15B/C (281).

Figure 7. Definition of pneumococcal serotype carriage episode

In this example, the individual first carries 6B and subsequently acquires 19F. Following clearance, there is reacquisition of serotype 6B. A = acquisition; B = clearance; C = carriage duration.



3.1.4.4. Data analysis

Non-normally distributed continuous data were described by median and inter-quartile range. Comparisons were made using the Wilcoxon rank-sum test. Categorical data were analysed by the chi-squared test. Logistic regression models, with either study participant identifier included as a random-effect or robust standard errors to control for repeated observations within individuals, were used to determine temporal changes in serotype distributions, multiple serotype detection, and transmission. Odds ratios, adjusted for the number of NPS specimens per individual, were calculated to determine the likelihood of carriage of serotypes in infants or mothers. Generalised Estimating Equations

(GEE), with a logistic link and exchangeable correlation structure to account for repeated observations per individual, were used to identify the risks of reacquisition of commonly carried serotypes and to determine the risks of acquisition of drug resistant pneumococci in infants (120). Assessments of GEE model fit were made using a modification to the Akaike information criterion (AIC) (282). Time to pneumococcal acquisition and carriage duration were estimated by survival analysis, since some carriage episodes were censored. The log-rank test was used to compare groups. Survival models (Cox proportional hazards or parametric models using exponential or Weibull distributions) were used to assess potential predictors of acquisition and carriage duration (283). Model fit was assessed by examination of predicted Cox-Snell residuals, the AIC, and log-likelihood values.

3.1.5. Results

Of 999 pregnant women recruited into the pneumonia cohort study, 250 were randomly allocated to the immunology follow-up group. Nineteen women and their babies did not attend the first follow-up visit (14 lost to follow-up, four neonatal deaths, and one stillbirth) resulting in 231 mothers and 234 infants (three sets of twins) who had at least one surveillance NPS collected. These 234 mother-infant pairs are included in the subsequent analyses.

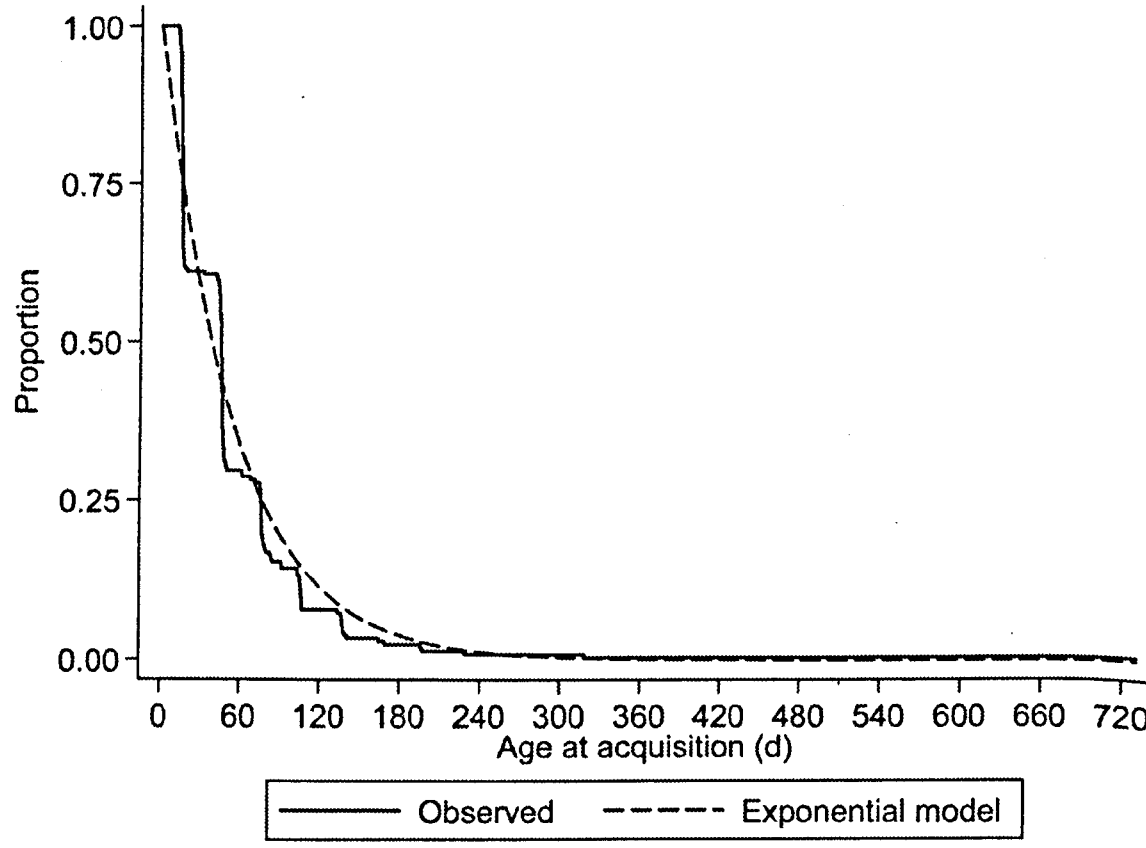
8,386 NPS were collected (73.6% of expected): 4,195 from mothers and 4,191 from infants (median 23 per individual; range 1 – 24 for infants, 2 – 25 for mothers). The median mother-infant pair follow-up duration was 23.9 months post-delivery (728 days; range 28 – 760). Overall there were 2,188 pneumococcal carriage episodes identified (1,504 infant; 684 mother): infants had a median of seven (range 0 – 15) carriage episodes, compared with a median of two (range 0 – 16) in mothers ($P < .001$).

3.1.5.1. Acquisition dynamics in infants

Infant acquisition was extremely rapid: by the three month visit 75.7% of infants had been colonised, increasing to 97.0% by six months. All but one infant remaining in the

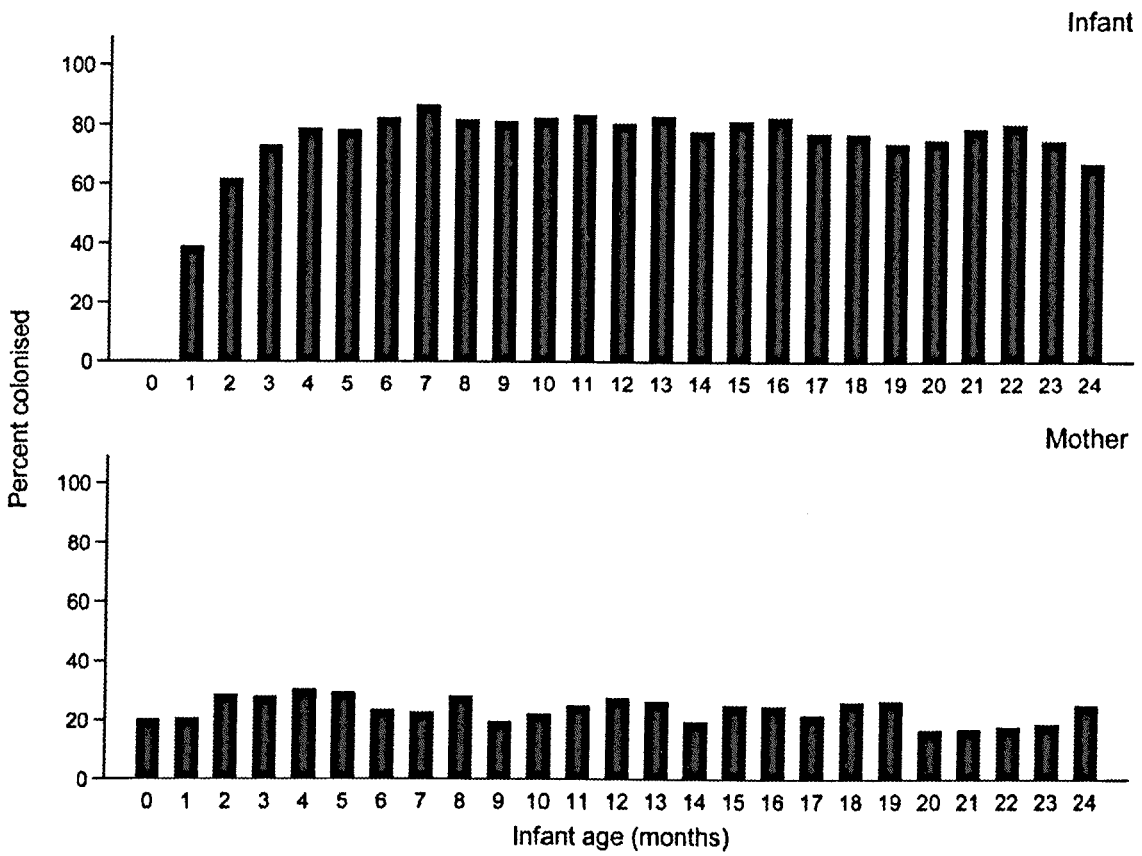
study had acquired pneumococci at least once by the eleven month visit. The median age of first acquisition was 46 days (95% CI 45 – 46), with an observed and modelled acquisition rate of 0.018 per day (Figure 8).

Figure 8. Observed and modelled age at first pneumococcal acquisition in the cohort of infants



Carriage stabilised after seven months and point-prevalence remained between 67.6 and 83.6% (Figure 9, top panel). Twenty percent of mothers were colonised by pneumococcus at delivery (+/- 7 days). This proportion increased after birth, peaking at 30.6% at the four month visit and subsequently stabilising with monthly colonisation point-prevalence of 16.8 - 29.6% (Figure 9, bottom panel).

Figure 9. Cross-sectional pneumococcal carriage prevalence in infants and mothers by month of infant age



Potential associations between age at first acquisition and household size, presence of other young children in the house, ethnic group, prematurity (delivery <37 weeks gestation), home delivery, season of birth, maternal pneumococcal colonisation at birth, maternal smoking, or antibiotic consumption in the neonatal period were explored (Table 10). The impact of feeding differences on colonisation age could not be assessed since breast feeding was almost universal. By univariate analysis, large households (>5 people), other children <5y in the house, mothers who smoked, and home delivery were all associated significantly with earlier acquisition age. In a multivariate Cox model, the presence of other children <5y in the house and maternal smoking remained associated significantly with an earlier age at first colonisation (children <5y: HR 1.47, $P = .01$; maternal smoking: HR 1.53, $P = .01$). Premature birth was associated with delayed

colonisation (HR 0.54, $P = .04$). There was a trend for female infants to become colonised at an earlier age than males (HR 1.34, $P = .05$).

Table 10. Univariate and multivariate Cox regression analyses of potential factors influencing age at first pneumococcal acquisition in infants

A Hazard Ratio >1 indicates earlier age at first acquisition. All factors were included in the multivariate model.

Factor	Univariate model		Multivariate model	
	Hazard Ratio (95% CI)	*P*	Hazard Ratio (95% CI)	*P*
Household size >5 people	1.34 (1.02 – 1.76)	.04	1.20 (0.89 – 1.62)	.2
Children <5y in the house	1.53 (1.17 – 2.01)	.002	1.47 (1.09 – 2.01)	.01
Mother smoker	1.63 (1.21 – 2.19)	.001	1.53 (1.10 – 2.14)	.01
Ethnic group:				
Sgaw Karen	-		-	
Pwo Karen	0.79 (0.51 – 1.24)	.3	0.89 (0.55 – 1.44)	.6
Muslim	1.12 (0.78 – 1.75)	.5	1.29 (0.84 – 1.98)	.3
Other	1.38 (0.56 – 3.36)	.5	1.94 (0.77 – 4.90)	.2
Season of birth:				
Hot (March – May)	-		-	
Wet (June – October)	0.75 (0.52 – 1.06)	.1	0.85 (0.58 – 1.24)	.4
Cool (November – February)	1.08 (0.75 – 1.54)	.7	1.13 (0.77 – 1.66)	.5
Home delivery	1.56 (1.13 – 2.16)	.007	1.23 (0.86 – 1.78)	.3
Prematurity	0.61 (0.38 – 1.00)	.05	0.54 (0.29 – 0.97)	.04
Female gender	1.20 (0.92 – 1.56)	.2	1.34 (1.00 – 1.79)	.05
Mother colonised at birth	1.28 (0.92 – 1.77)	.1	1.21 (0.86 – 1.72)	.3
Antibiotics in neonatal period	0.79 (0.52 – 1.22)	.3	0.95 (0.58 – 1.55)	.8

Age at first acquisition was not significantly different between the commonest eight serotypes or between grouped vaccine (PCV13) serotypes, non-vaccine serotypes (NVT), and non-typeable (NT) pneumococci (Table 11). Estimates of individual serotype acquisition rates, excluding serotype reacquisitions, over the entire cohort follow-up period are summarised in Table 12 and Figure 10.

3.1.5.2. Pneumococcal carriage kinetics

The median duration of the infants' first ever carriage episode was 63 days (95% CI 61 – 90). Duration of carriage of serotypes 19F and 23F was longer ($P < .001$ and .008 respectively), and NT pneumococci shorter ($P = .01$), than for the other serotypes (Table 11). Following the first carriage episode, infants had 1,279 subsequent pneumococcal acquisitions: 620 in the first year of life and 659 in the second. NT pneumococci, 19F, 23F, and 6B remained the most commonly carried serotypes (Table 11). Median age at second pneumococcal serotype acquisition was 111 days (95% CI 107 – 137), with no significant differences between PCV13 serotypes, NVT, and NT pneumococci. The median interval between acquisitions was 62 days (95% CI 61 – 63). Considering all subsequent carriage episodes, PCV13 serotypes were carried for longer than NVT and NT pneumococci (medians 62, 46, and 34 days; $P < .001$), although there was substantial variation between individual serotypes (Table 11). Estimates of serotype specific clearance rates following primary acquisitions of the serotype are summarised in Table 12 and Figure 10. For serotypes with at least ten primary acquisitions, acquisition rates were correlated with prevalence ($r = 0.99$, $P < .001$) and log rates of acquisition and clearance were negatively correlated ($r = -0.69$, $P < .001$) (Figure 11).

Table 11. Age at acquisition and duration of pneumococcal carriage

Ten commonest serotypes, ranked by number of carriage episodes.

Infant: first episode				Infant: all subsequent episodes			Mother: all episodes		
Serotype	**N**	**Acquisition age, d Median (95% CI)**	**Carriage duration , d Median (95% CI)**	**Serotype**	**N**	**Carriage duration , d Median (95% CI)**	**Serotype**	**N**	**Carriage duration, d Median (95% CI)[a]**
NT	35	46 (16 - 48)	31 (31 - 61)	**NT**	160	34 (32 - 60)	**NT**	145	32 (31 - 51)
19F	23	47 (16 - 80)	213 (78 - 243)	**19F**	146	92 (62 - 120)	**19F**	50	31 (30 - 31)
23F	21	45 (15 - 47)	184 (62 - 241)	**23F**	114	90 (62 - 94)	**23F**	31	31 (30 - 32)
6B	14	44 (16 - 48)	120 (61 - 153)	**6B**	105	62 (58 - 91)	**34**	25	31 (30 - 59)
35F	9	46[b]	121 (30 - 180)	**14**	72	87 (61 - 92)	**3**	23	46 (31 - 90)
11A	8	46 (16 - 77)	60 (30 - 90)	**15B/C**	68	61 (49 - 91)	**6B**	23	31 (30 - 32)
14	8	16 (13 - 83)	62 (30 - 151)	**6A**	60	63 (48 - 121)	**14**	22	32 (31 - 60)
28F	8	46 (14 - 138)	61 (30 - 63)	**6C**	46	62 (33 - 94)	**11A**	18	33 (30 - 48)
4	7	16 (13 - 20)	84[b]	**19A**	43	59 (31 - 63)	**35C**	18	32 (30 - 33)
34	5	16[b]	123[b]	**34**	38	63 (32 - 134)	**15B/C**	16	32 (29 - 32)
PCV13	95	44 (16 - 47)	116 (88 - 127)	**PCV13**	611	62 (61 - 76)	**PCV13**	223	31 (31 - 32)
NVT[c]	95	46 (44 - 46)	61 (57 - 87)	**NVT[c]**	508	46 (33 - 58)	**NVT[c]**	316	31 (31 - 32)
All	225	46 (44 - 46)	63 (61 - 90)	**All**	1,279	60 (57 - 61)	**All**	684	31 (31 - 32)

[a] Duration calculated for 602 carriage episodes (acquisition date could not be calculated for carriage episodes including the first or second swab)
[b] Unable to estimate 95% CI
[c] Excluding non-typeable pneumococci

Table 12. Pneumococcal acquisition and clearance rates in infants

Time to first acquisition and subsequent clearance of the serotype (includes each infant's first episode of carriage for each serotype). Results for all serotypes with >10 acquisitions.

Serotype	Episodes	Acquisition		Clearance	
	(N)	Rate (/day)	95% CI	Rate (/day)	95% CI
NT	129	0.00164	(0.00138 - 0.00195)	0.01351	(0.01124 - 0.01623)
19F	111	0.00133	(0.00110 - 0.00160)	0.00608	(0.00497 - 0.00744)
23F	101	0.00110	(0.00091 - 0.00134)	0.00666	(0.00535 - 0.00829)
6B	85	0.00087	(0.00071 - 0.00108)	0.01007	(0.00808 - 0.01255)
14	71	0.00067	(0.00053 - 0.00085)	0.00932	(0.00716 - 0.01214)
15B/C	65	0.00058	(0.00046 - 0.00075)	0.01058	(0.00799 - 0.01399)
6A	55	0.00049	(0.00037 - 0.00063)	0.00747	(0.00536 - 0.01040)
6C	43	0.00038	(0.00028 - 0.00051)	0.01099	(0.00796 - 0.01516)
19A	42	0.00036	(0.00027 - 0.00049)	0.01040	(0.00743 - 0.01456)
34	38	0.00032	(0.00024 - 0.00044)	0.00843	(0.00574 - 0.01239)
15A	28	0.00023	(0.00016 - 0.00034)	0.01284	(0.00846 - 0.01950)
13	25	0.00021	(0.00014 - 0.00030)	0.01588	(0.01001 - 0.02521)
38	23	0.00019	(0.00012 - 0.00028)	0.01744	(0.01068 - 0.02847)
10B	22	0.00018	(0.00012 - 0.00028)	0.01726	(0.01126 - 0.02648)
35C	22	0.00018	(0.00012 - 0.00028)	0.01367	(0.00882 - 0.02120)
18C	20	0.00016	(0.00011 - 0.00025)	0.02093	(0.01335 - 0.03281)
11A	19	0.00016	(0.00010 - 0.00025)	0.01976	(0.01245 - 0.03136)
9V	18	0.00015	(0.00009 - 0.00023)	0.01309	(0.00802 - 0.02137)
21	18	0.00015	(0.00009 - 0.00023)	0.01319	(0.00820 - 0.02122)
23A	18	0.00015	(0.00009 - 0.00024)	0.01264	(0.00775 - 0.02064)
33B	17	0.00014	(0.00009 - 0.00022)	0.01009	(0.00608 - 0.01674)
9N	16	0.00013	(0.00008 - 0.00021)	0.01742	(0.01050 - 0.02890)
28F	16	0.00013	(0.00008 - 0.00021)	0.01693	(0.00983 - 0.02915)
35F	16	0.00013	(0.00008 - 0.00022)	0.01021	(0.00616 - 0.01694)
19B	15	0.00012	(0.00007 - 0.00020)	0.01563	(0.00908 - 0.02693)
3	14	0.00011	(0.00007 - 0.00019)	0.01829	(0.01039 - 0.03221)
10A	14	0.00011	(0.00007 - 0.00019)	0.00989	(0.00548 - 0.01785)
4	13	0.00011	(0.00006 - 0.00018)	0.01662	(0.00920 - 0.03000)
17F	13	0.00011	(0.00006 - 0.00018)	0.01844	(0.01071 - 0.03176)
22A	12	0.00010	(0.00006 - 0.00017)	0.01789	(0.00991 - 0.03230)
16F	11	0.00009	(0.00005 - 0.00016)	0.02356	(0.01268 - 0.04378)
PCV13	204	0.00607	(0.00530 - 0.00697)	0.00805	(0.00696 - 0.00932)
NVT[a]	191	0.00465	(0.00404 - 0.00536)	0.01288	(0.01110 - 0.01495)
All[b]	221	0.01837	(0.01610 - 0.02096)	0.00962	(0.00839 - 0.01104)

[a] Excluding non-typeable pneumococci

[b] 211/234 infants acquired pneumococci during follow-up: acquisition and clearance of first pneumococcus

Figure 10. Serotype-specific acquisition and clearance rates in infants over the entire follow-up period

Serotype reacquisitions were excluded. Results for all serotypes with >10 acquisitions.

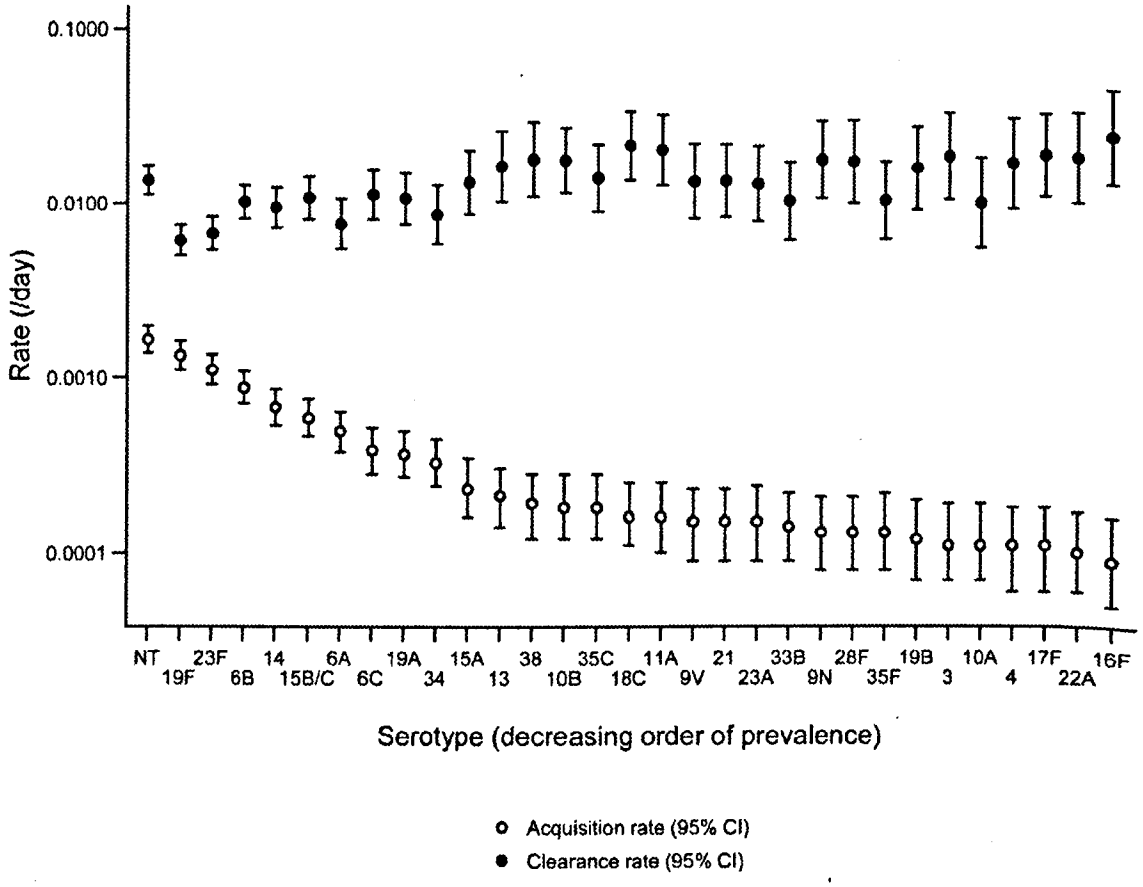
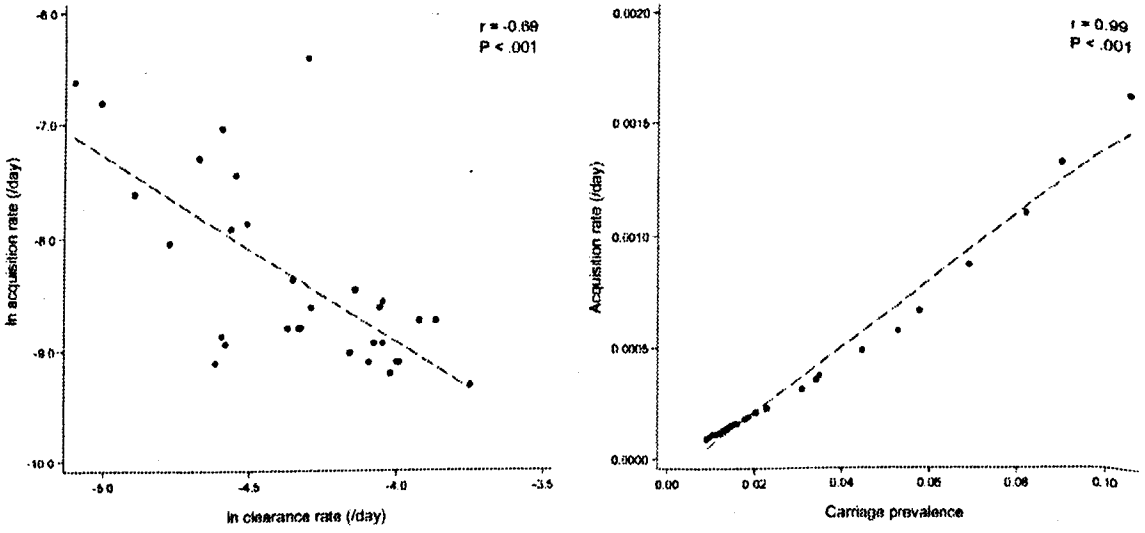


Figure 11. Scatter plots of serotype-specific prevalence, acquisition, and clearance rates



There were 272 instances of re-acquisition of a previously carried serotype. NT pneumococci, 19F, 23F, 6B and 14 accounted for ~75% of these re-acquisitions.

A Generalised Estimating Equation (GEE), with a logistic link and exchangeable correlation structure, was used to identify the risks of reacquisition of the ten most commonly carried serotypes (6A, 6B, 6C, 14, 15B/C, 19F, 19A, 23F, 34, and NT) following previous carriage of the homologous or heterologous serotypes. Infants were considered to be at-risk of acquisition of a serotype if this serotype was not present in either of the two preceding NPS specimens. In the first instance, crude odds ratios were calculated for the risk of reacquiring a previously carried serotype. Subsequently, a full model was fitted which included previous carriage of all of these serotypes, presence of any other pneumococcal serotype in the preceding NPS, antimicrobial use in the preceding 30 days, plus household factors (large household (>5 people) and presence of other children <5y in the house). In the crude assessment, a protective effect was not observed for any of the serotypes assessed. Conversely, infants with previous carriage of serotypes 6A/B/C, 19F, 23F, or 34 had a significantly greater risk of subsequent reacquisition than those who had not been colonised (Table 13). With the exception of previous carriage of 19F resulting in a reduced risk of acquisition of 6C (OR 0.45, $P = .046$), no serotype-specific protective effects of previous colonisation were found. The presence of a pneumococcus of any other serotype in the preceding NPS was protective against acquisition of 6B (OR 0.66, $P = .048$), 6C (OR 0.32, $P < .001$), 19A (OR 0.47, $P = .02$), and 23F (OR 0.66, $P = .04$). As in the crude analysis, infants with previous colonisation by 6B (OR 3.00, $P < .001$), 19F (OR 2.65, $P < .001$), 23F (OR 2.65, $P < .001$), and 34 (OR 3.85, $P = .002$) had significantly higher risk of subsequent colonisation by the homologous serotype than non-colonised infants (Table 14). For serotypes 6B, 6C, and 19F the presence of additional young children in the house was a risk factor for acquisition (6B: OR 1.55, 95% CI 1.04 – 2.32, $P = .03$; 6C: OR 2.21, 95% CI 1.16 – 4.21, $P = .02$; 19F: OR 1.47, 95% CI 1.03 – 2.10, $P = .03$). Antimicrobial consumption in the preceding 30 days

was protective against colonisation by 6A (OR 0.12, 95% CI 0.02 – 0.89, $P = .04$) and a risk factor for 19F acquisition (OR 1.69, 95% CI 1.09 – 2.63, $P = .02$).

The effect of previous colonisation by homologous and heterologous serotypes on timing and duration of reacquisitions of serotypes 6B, 14, 19F, 23F, and NT pneumococci was examined. Controlling for age and carriage of heterologous serotypes, previous carriage of serotype 14 or 19F was associated with significantly reduced duration (HR >1) of subsequent carriage episodes of the same serotype, and there was a trend in the same direction for serotype 23F. Previous carriage was also associated with an increased interval to reacquisition (HR <1) of the same serotype (Table 15). Previous colonisation by heterologous serotypes also impacted on time to reacquisition of these five serotypes (HR <1, $P < .05$ for all serotypes), but only for 19F could a reduction of carriage duration be demonstrated (HR 2.01, $P = .01$).

Table 13. Crude risk of pneumococcal serotype acquisition if previous colonisation by the homologous serotype

	Serotype									
	6A	**6B**	**6C**	**14**	**15B/C**	**19F**	**19A**	**23F**	**34**	**NT**
OR (95% CI) for acquisition following previous colonisation by the homologous serotype	2.28 (1.11 - 4.67)	2.09 (1.36 - 3.21)	2.60 (1.22 - 5.62)	1.33 (0.72 - 2.45)	1.65 (0.84 - 3.21)	1.56 (1.08 - 2.26)	1.59 (0.61 - 4.17)	1.59 (1.04 - 2.43)	3.07 (1.32 - 7.10)	0.97 (0.68 - 1.37)
P	0.03	0.001	0.01	0.4	0.1	0.02	0.3	0.03	0.009	0.8
% of at-risk specimens positive for new acquisition (no. at-risk specimens):										
0 previous acquisitions	1.48 (3,713)	2.66 (3,198)	1.14 (3,756)	2.04 (3,474)	1.78 (3,643)	4.01 (2,766)	1.11 (3,790)	3.34 (3,028)	0.99 (3,844)	4.94 (2,612)
≥1 previous acquisitions	3.09 (259)	6.00 (567)	2.56 (273)	1.97 (458)	1.81 (332)	7.99 (713)	1.5 (253)	5.67 (600)	2.39 (209)	6.20 (1,048)

Table 14. Adjusted analysis of the effect of previous serotype colonisation on new acquisitions of heterologous or homologous serotypes

Newly acquired serotype	Previously carried serotype										
	6A	6B	6C	14	15B/C	19F	19A	23F	34	NT	Prev. swab grew Pnc[b]
6A	**1.44**	0.97	2.04	1.99[a]	2.49[a]	1.73	0.91	1.20	1.17	1.48	0.69
	(0.62 - 3.31)	(0.51 - 1.86)	(0.96 - 4.33)	(1.05 - 3.77)	(1.24 - 4.99)	(0.94 - 3.15)	(0.37 - 2.23)	(0.64 - 2.24)	(0.48 - 2.87)	(0.84 - 2.61)	(0.39 – 1.22)
6B	2.03[a]	**3.00**[a]	2.19[a]	0.70	2.37[a]	1.78[a]	0.80	1.36	0.59	0.89	0.66[a]
	(1.11 - 3.71)	(1.82 - 4.93)	(1.17 - 4.12)	(0.38 - 1.28)	(1.37 - 4.09)	(1.12 - 2.84)	(0.37 - 1.71)	(0.84 - 2.19)	(0.25 - 1.39)	(0.57 - 1.38)	(0.44 – 0.99)
6C	0.35	0.71	**1.49**	1.23	0.71	0.45[a]	0.99	1.90	1.48	2.58	0.32[a]
	(0.08 - 1.50)	(0.32 - 1.59)	(0.59 - 3.76)	(0.55 - 2.76)	(0.26 - 1.95)	(0.21 - 0.99)	(0.36 - 2.74)	(0.94 - 3.84)	(0.54 - 4.03)	(1.35 - 4.92)	(0.18 – 0.58)
14	2.52[a]	1.99[a]	1.13	**0.53**	0.54	1.42	1.16	1.66	0.86	1.04	0.86
	(1.29 - 4.94)	(1.13 - 3.48)	(0.50 - 2.54)	(0.23 - 1.21)	(0.22 - 1.34)	(0.81 - 2.49)	(0.50 - 2.65)	(0.95 - 2.90)	(0.33 - 2.23)	(0.62 - 1.73)	(0.51 – 1.45)
15B/C	0.90	0.96	1.15	0.75	**0.52**	1.06	1.80	0.89	1.53	1.44	1.23
	(0.40 - 2.05)	(0.52 - 1.75)	(0.55 - 2.40)	(0.36 - 1.53)	(0.20 - 1.34)	(0.60 - 1.89)	(0.88 - 3.65)	(0.49 - 1.62)	(0.70 - 3.37)	(0.86 - 2.42)	(0.68 – 2.21)
19F	1.10	1.65[a]	1.51	0.92	0.68	**2.65**[a]	0.50	1.58[a]	1.15	1.17	0.82
	(0.60 - 2.01)	(1.07 - 2.56)	(0.85 - 2.68)	(0.55 - 1.54)	(0.35 - 1.32)	(1.69 - 4.14)	(0.21 - 1.17)	(1.04 - 2.40)	(0.59 - 2.25)	(0.80 - 1.70)	(0.58 – 1.17)
19A	1.35	1.36	1.49	1.50	1.37	1.13	**0.60**	2.45[a]	1.95	1.01	0.47[a]
	(0.52 - 3.52)	(0.62 - 2.95)	(0.52 - 4.28)	(0.65 - 3.47)	(0.54 - 3.45)	(0.54 - 2.37)	(0.14 - 2.55)	(1.19 - 5.04)	(0.76 - 5.02)	(0.51 - 2.03)	(0.25 – 0.87)
23F	1.05	1.35	1.04	1.11	0.71	1.74[1]	1.55	**2.65**[a]	0.67	1.16	0.66[a]
	(0.55 - 2.02)	(0.84 - 2.18)	(0.52 - 2.10)	(0.65 - 1.90)	(0.35 - 1.42)	(1.11 - 2.74)	(0.82 - 2.94)	(1.67 - 4.21)	(0.27 - 1.67)	(0.77 - 1.74)	(0.45 – 0.97)
34	2.38[a]	0.88	1.05	1.74	2.02	1.26	0.91	2.01[a]	**3.85**[a]	1.44	0.53
	(1.06 - 5.30)	(0.40 - 1.94)	(0.36 - 3.04)	(0.79 - 3.86)	(0.89 - 4.61)	(0.61 - 2.60)	(0.31 - 2.67)	(1.03 - 4.08)	(1.64 - 9.06)	(0.77 - 2.87)	(0.27 – 1.05)
NT	0.71	1.26	1.19	1.09	1.44	1.28	1.48	1.12	0.62	**0.90**	1.05
	(0.37 - 1.38)	(0.82 - 1.92)	(0.66 - 2.13)	(0.67 - 1.75)	(0.85 - 2.44)	(0.85 - 1.94)	(0.84 - 2.59)	(0.73 - 1.71)	(0.28 - 1.37)	(0.60 - 1.34)	(0.75 – 1.46)

Results summarised as odds ratios and 95% confidence intervals; boldface values represent homologous serotype comparisons

[a] $P < .05$; [b] Nasopharyngeal swab from the preceding month grew any other pneumococcal serotype

Table 15. Effect of previous carriage on reacquisition and carriage duration of common pneumococcal serotypes

First episodes of serotype carriage, not necessarily the infant's first ever carriage episode, were compared with subsequent episodes of carriage of the same serotype, controlling for age and carriage of heterologous serotypes.

Serotype	No. carriage episodes (first / reacq.)	Reacquisition number	Carriage duration		Time to reacquisition[b]	
			HR (95% CI)[a]	*P*	HR (95% CI)[c]	*P*
NT	195 (129/66)	1st	1.13 (0.79 - 1.63)	.5	0.18 (0.10 - 0.35)	< .001
		2nd	0.75 (0.33 - 1.71)	.5	0.22 (0.07 - 0.63)	.005
		3rd	0.31 (0.04 - 2.46)	.3	0.40 (0.12 - 1.38)	.1
19F	169 (112/57)	1st	2.64 (1.50 - 4.67)	.001	0.39 (0.14 - 1.08)	.07
		2nd	2.81 (1.09 - 7.22)	.03	0.35 (0.09 - 1.32)	.1
		3rd	1.39 (0.90 - 2.16)	.1	1.00 (0.19 - 5.16)	1.0
23F	135 (101/34)	1st	1.56 (0.94 - 2.60)	.08	0.50 (0.19 - 1.31)	.2
		2nd	3.20 (1.93 - 5.28)	< .001	0.19 (0.04 - 0.95)	.04
		3rd	0.76 (0.51 - 1.11)	.2	0.79 (0.08 - 7.99)	.8
6B	119 (85/34)	1st	1.18 (0.62 - 2.23)	.6	0.30 (0.11 - 0.84)	.02
		2nd	1.21 (0.59 - 2.46)	.6	0.21 (0.05 - 0.84)	.03
		3rd	-	-	-	-
14	80 (71/9)	1st	4.12 (1.35 - 12.59)	.01	0.04 (0.04 - 0.35)	.004
		2nd	-	-	-	-
		3rd	-	-	-	-

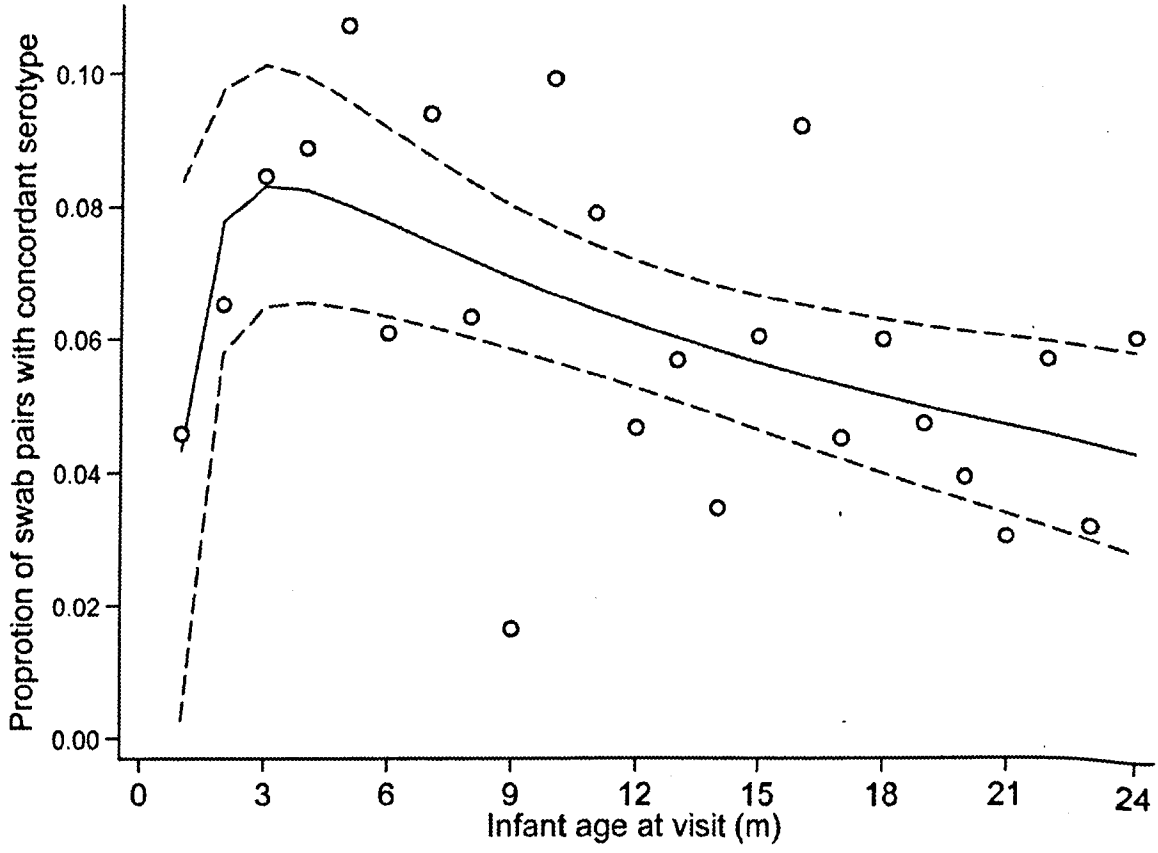
[a] Cox proportional hazards model
[b] Time from clearance of a serotype to subsequent reacquisition
[c] Parametric survival model (Weibull distribution)

The pneumococcal nasopharyngeal acquisition rate was significantly lower in mothers compared with infants (0.5 vs. 1.1 per 100 person-days; $P < .001$). Mothers carried pneumococci for a shorter duration than infants (median 31 vs. 61 days; $P < .001$) (Table 11).

There were 3,963 mother-infant swab pairs (swabs taken from both mother and infant on the same day), and in 233 (5.9%) a common serotype was identified. Forty six of the mothers were colonised by pneumococci at delivery and only ten (21.7%) of the infants acquired the mother's serotype as their first colonising pneumococcus. Identification of a

common serotype in a mother-infant pair became less common as the infant's age increased (for each one month increase: OR 0.98, 95% CI 0.96 – 0.99, $P = .02$) which, along with an increasing proportion of colonised mothers in the four months following delivery, suggests transmission occurred more frequently in the early months of life (Figure 12).

Figure 12. Proportion of mother-infant NPS pairs in which a common pneumococcal serotype was identified, by infant age



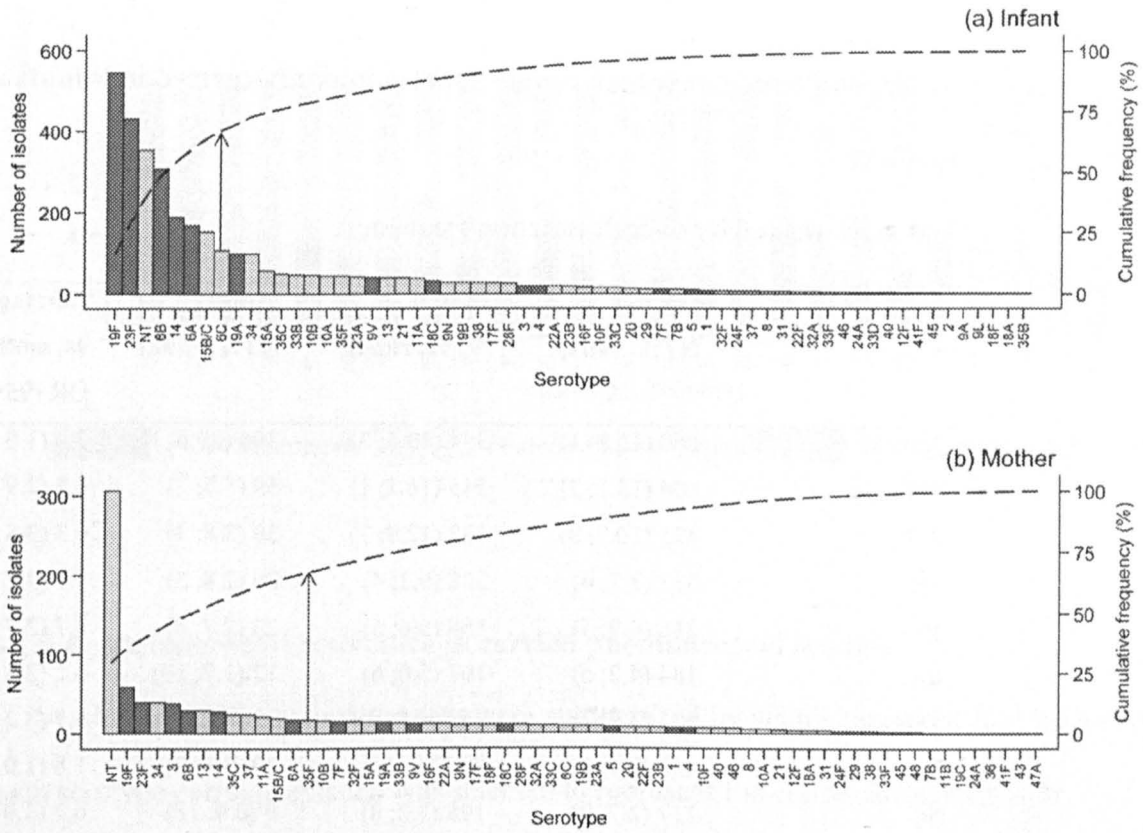
3.1.5.3. Pneumococcal serotype distribution

A total of 4,396 pneumococci were isolated from all swabs taken, comprising 67 serotypes (3,363 from infants; 1,033 from mothers). In infants, eight serotypes (19F, 23F, NT, 6B, 14, 6A, 15B/C, 6C) accounted for 67.0% of the isolates (Figure 13(a)). In

mothers, 29.6% of isolates were non-typeable with no other predominant serotypes (Figure 13(b)).

Figure 13. Pneumococcal serotype distribution in infants and mothers

The bars indicate the number of isolates of each serotype and the dashed lines indicate the cumulative frequency, with a cumulative frequency of 67% is indicated by the vertical arrows. Dark grey bars highlight PCV13 serotypes.



Over half (55.8%) of pneumococci from infants were PCV13 serotypes, compared with 27.5% from mothers ($P < .001$) (Table 16). The proportion of pneumococci that were PCV13 serotypes increased significantly over the first year of life in infants (from 46.2% at 1 month to 64.4% at 12 months; $P < .001$) and then decreased over the second year (Figure 14). Infants carried a median of five (range 0 – 13), and mothers a median of two (range 0 – 11), serotypes over the 24 month observation period. Considering serotype carriage over

the entire follow-up period (as binary "yes/no" variables for carriage of each serotype in an individual), all of the most common serotypes were more likely to be carried by infants than mothers, controlling for the number of NPS collected per individual in a logistic regression model (OR >1, Table 16). In swabs where pneumococci were cultured, multiple serotypes were identified in 5.1% of infant swabs and 2.6% of mother swabs. Detection of more than one carried serotype became more common as age increased (infants $P < .001$; mothers $P = .001$).

Table 16. Pneumococcal serotypes most commonly carried in infants and their mothers

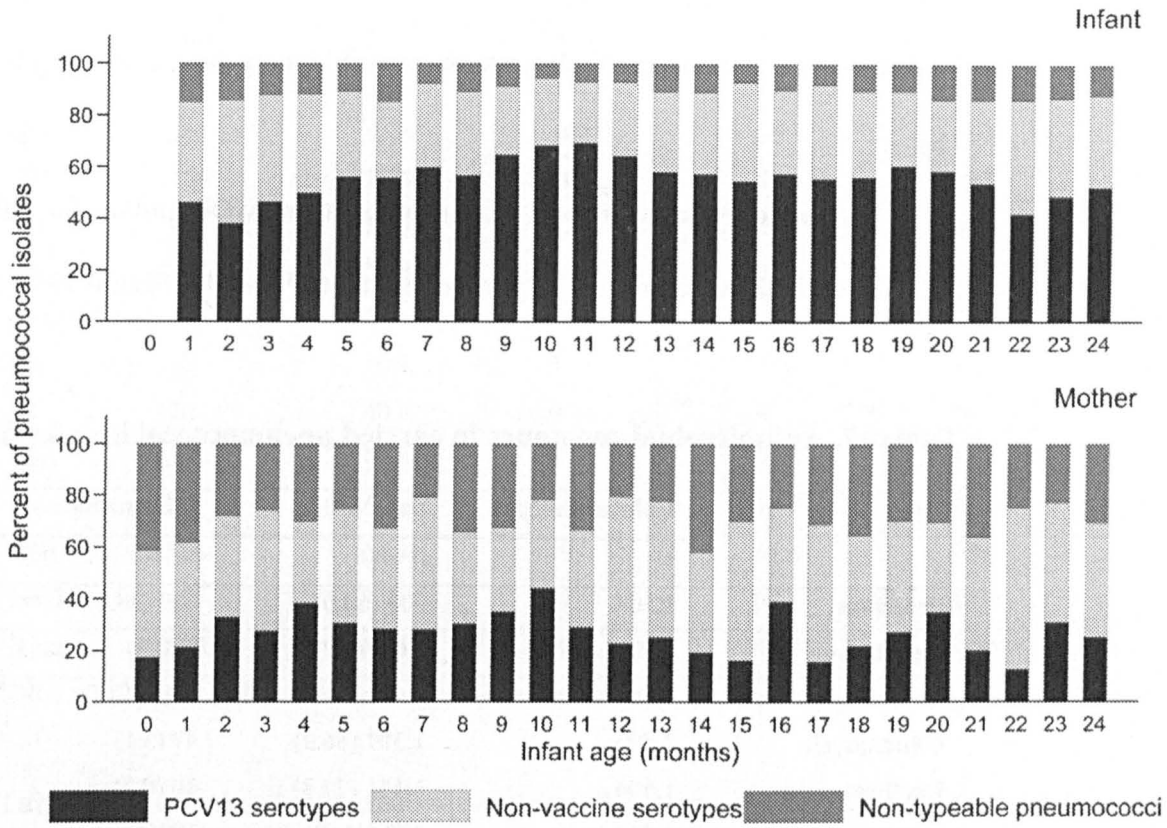
Serotypes ranked by overall isolation frequency.

Serotype	Overall N (%; rank)	Infant N (%; rank)	Mother N (%; rank)	Carriage in infants vs. mothers OR (95% CI)[a]
NT	660 (15.0; 1)	354 (10.5; 3)	306 (29.6; 1)	2.8 (1.9 - 4.2)
19F	604 (13.7; 2)	545 (16.2; 1)	59 (5.7; 2)	4.5 (2.9 - 7.0)
23F	471 (10.7; 3)	432 (12.9; 2)	39 (3.8; 3)	6.3 (3.8 - 10.2)
6B	337 (7.7; 4)	308 (9.2; 4)	29 (2.8; 5)	7.3 (4.2 - 12.8)
14	216 (4.9; 5)	188 (5.6; 5)	28 (2.7; 6)	4.7 (2.7 - 8.0)
6A	184 (4.2; 6)	167 (5.0; 6)	17 (1.7; 10)	5.2 (2.8 - 9.7)
15B/C	171 (3.9; 7)	152 (4.5; 7)	19 (1.8; 9)	5.7 (3.2 - 10.4)
34	138 (3.1; 8)	99 (2.9; 10)	39 (3.8; 3)	1.8 (1.0 - 3.2)
6C	117 (2.7; 9)	108 (3.2; 8)	9 (0.9; 18)	6.7 (3.0 - 14.7)
19A	114 (2.6; 10)	100 (3.0; 9)	14 (1.4; 13)	3.9 (2.0 - 7.6)
PCV13 serotypes	2,162 (49.2%)	1,878 (55.8%)	284 (27.5%)	9.0 (5.1 - 15.7)
NVT serotypes[b]	1,574 (35.8%)	1,131 (33.6%)	443 (42.9%)	8.3 (4.4 - 15.6)
Total	4,396	3,363	1,033	4.9 (2.3 - 10.2)

[a] OR for the serotype being carried at any time point (adjusted for number of swabs per individual; all $P < .05$)

[b] Excluding non-typeable pneumococci

Figure 14. Pneumococcal isolates by serotype category in infants and mothers, by infant age



3.1.5.4. Antimicrobial resistance in carried pneumococcal isolates

Antimicrobial resistance data were summarised by acquisitions: the first isolate of each serotype carriage episode was selected to represent the entire carriage episode. Therefore, 1,504 pneumococci from infant carriage episodes and 546 pneumococci from mother carriage episodes were analysed. Pneumococci were determined to be multi-drug resistant if they were resistant to three of more of chloramphenicol, erythromycin, penicillin (MIC $\geq$0.12 μg/mL), tetracycline, or trimethoprim-sulphamethoxazole (co-trimoxazole).

Forty percent of infant isolates were penicillin non-susceptible and 34.6% were MDR. PCV13 serotypes were significantly more likely to be MDR ($P < .001$). Over 75% of infant isolates were fully susceptible to chloramphenicol, clindamycin, and

erythromycin whereas only 21.3% and 40.5% of isolates were fully susceptible to co-trimoxazole and tetracycline, respectively (Table 17). Of the MDR isolates, over two-thirds were from four PCV13 serotypes (6B, 19F, 19A, and 23F). Whilst serotype 14 and NT isolates were frequently penicillin non-susceptible, they were less likely to be MDR (Table 18).

A quarter of pneumococci from mothers were MDR and, as for infant isolates, PCV13 serotypes were more likely to be resistant ($P < .001$) (Table 19 & Table 20).

Table 17. Antimicrobial resistance in carried pneumococcal isolates from infants

Drug	Isolates tested[a]	Susceptible	Intermediate	Resistant
	N	N (%)	N (%)	N (%)
Penicillin	1,504	903 (60.0)	580 (38.6)	21 (1.4)
Ceftriaxone[b]	695	693 (99.7)	2 (0.3)	0
Chloramphenicol	1,504	1,406 (93.5)	NA[c]	98 (6.5)
Clindamycin	1,504	1,307 (86.9)	47 (3.1)	150 (10.0)
Erythromycin	1,504	1,171 (77.8)	10 (0.7)	323 (21.5)
Co-trimoxazole	1,504	320 (21.3)	197(13.1)	987 (65.6)
Tetracycline	1,504	609 (40.5)	69 (4.6)	826 (54.9)

[a] Reporting a single isolate per pneumococcal serotype carriage episode
[b] Tested if oxacillin screening disc <20 mm
[c] There is no intermediate susceptibility category for chloramphenicol

Table 18. Antimicrobial resistance in the most commonly carried pneumococcal serotypes from infants

Serotype	MDR		Penicillin non-susceptible[a]		Total
	N	%	N	%	
NT	75	38.5	157	80.5	195
19F	161	95.3	162	95.9	169
23F	104	77.0	98	72.6	135
6B	63	52.9	33	27.7	119
14	13	16.3	58	72.5	80
15B/C	4	5.6	9	12.7	71
6A	13	20.6	3	4.8	63
6C	20	40.0	0	0	50
19A	39	84.8	43	93.5	46
34	2	4.7	8	18.6	43
PCV13	398	56.4	407	57.6	706
NVT[b]	47	7.8	37	6.1	603
Total	520	34.6	601	40.0	1,504

[a] MIC ≥0.12 μg/mL; [b] Excluding non-typeable pneumococci

Table 19. Antimicrobial resistance in carried pneumococcal isolates from mothers

Drug	Isolates tested[a]	Susceptible	Intermediate	Resistant
	N	N (%)	N (%)	N (%)
Penicillin	546	359 (65.8)	182 (33.3)	5 (0.9)
Ceftriaxone[b]	231	229 (99.1)	2 (0.9)	0
Chloramphenicol	546	514 (94.1)	NA[c]	32 (5.9)
Clindamycin	546	492 (90.1)	17 (3.1)	37 (6.8)
Erythromycin	546	464 (85.0)	5 (0.9)	77 (14.1)
Co-trimoxazole	546	163 (29.8)	73 (13.4)	310 (56.8)
Tetracycline	546	232 (42.5)	43 (7.9)	271 (49.6)

[a] Reporting a single isolate per pneumococcal serotype carriage episode
[b] Tested if oxacillin screening disc <20 mm
[c] There is no intermediate susceptibility category for chloramphenicol

Table 20. Antimicrobial resistance in the ten most commonly carried pneumococcal serotypes from mothers

Serotype	MDR		Penicillin non-susceptible[a]		Total
	N	%	N	%	
NT	45	43.7	77	74.8	103
19F	40	90.9	42	95.5	44
23F	20	71.4	19	67.9	28
34	1	4.3	1	4.3	23
3	0	0	0	0	20
6B	11	55.0	7	35.0	20
14	5	25.0	18	90.0	20
11A	0	0	0	0	17
35C	1	6.3	1	6.3	16
19A	10	90.9	11	100.0	11
PCV13	89	46.6	101	52.9	191
NVT[b]	13	5.2	9	3.6	252
Total	147	26.9	187	34.3	546

[a] MIC ≥0.12 μg/mL
[b] Excluding non-typeable pneumococci

The proportion of antimicrobial resistant isolates was greater in infants compared to mothers (MDR: 34.6% vs. 26.9%, $P = .001$; penicillin non-susceptible 40.0% vs. 34.3%, $P = .02$). This finding was also true when restricted to PCV13 serotypes: 56.4% of infant isolates were MDR compared to 46.6% of mother isolates ($P = .02$). There were no significant differences in resistance between infant and mother isolates for non-vaccine serotypes and non-typeable pneumococci.

Risk factors for acquisition of drug resistant pneumococci were explored using a generalised estimating equation with a logistic link. Correcting for the number of swabs collected per individual, only the presence of other young children in the house was associated significantly with a greater risk of acquisition of both multi-drug resistant and penicillin non-susceptible pneumococci ($P < .001$). Female infants were more likely to acquire penicillin non-susceptible, but not MDR, pneumococci than males ($P = .005$).

Acquisition of another pneumococcal serotype at the preceding NPS was protective against acquisition of a drug resistant pneumococcus ($P < .001$) (Table 21).

Table 21. Risk factors for acquisition of drug-resistant pneumococci in infants

Risk factor for acquisition	MDR			Penicillin non-susceptible		
	OR	(95% CI)	*P*	OR	(95% CI)	*P*
Female gender	1.15	(0.94 - 1.40)	.2	1.32	(1.09 - 1.60)	.005
Large household size (>5)	0.99	(0.81 - 1.22)	.9	1.09	(0.90 - 1.33)	.4
Children <5 y in house	1.59	(1.29 - 1.97)	< .001	1.47	(1.21 - 1.80)	< .001
Adults >60 y in house	1.18	(0.88 - 1.57)	.3	1.14	(0.87 - 1.50)	.3
Mother smokes	1.06	(0.86 - 1.31)	.6	1.01	(0.82 - 1.23)	1.0
One month age increase	1.00	(0.99 - 1.01)	1.0	1.00	(0.99 - 1.02)	.7
Pneumococcal serotype acquisition at preceding NPS	0.69	(0.56 - 0.85)	< .001	0.69	(0.57 - 0.84)	< .001
Antibiotics in previous 30 d:						
Any class	1.13	(0.85 - 1.50)	.4	-	-	-
Beta-lactam	-	-	-	1.24	(0.94 - 1.64)	.1

3.1.6. Discussion

To date, this is the largest longitudinal pneumococcal carriage study in children under two years, in terms of the number studied and the sampling frequency/duration of follow-up. Adherence to the WHO detection protocol enables direct comparison with other similar studies. The high level of follow-up and the uniformity of swab frequency over the follow-up period permitted confident assessment of carriage prevalence and dynamics. The inclusion of consistent follow-up in the second year of life was important since the risk of invasive disease remains high (119, 284), but there are few detailed data on pneumococcal carriage dynamics in children aged 12 - 24 months. Over 40% of all observed pneumococcal acquisitions occurred in the second year of life and there were significantly more re-acquisitions of previously carried serotypes in the second 12 months compared to the first 12 months of life, perhaps as a result of waning immunity.

In this crowded refugee camp, infants were colonised frequently by pneumococci and the first acquisition occurred early in life. The median age of acquisition (46 days) is similar to published studies from Asia and Africa, although in individual studies from Papua New Guinea and The Gambia, infants were colonised even earlier (60% by 15 days and 50% by 33 days respectively) (72, 73). The observed and modelled acquisition rate of 0.018 per day was identical to that found in a contemporary Kenyan birth cohort which used very short sampling intervals in the first weeks of life (74). In contrast, studies from the USA and Europe document older age at first pneumococcal acquisition, and lower overall carriage prevalence: the mean age at first acquisition was six months in the USA (47), and 56% of Finnish infants were colonised by 12 months with an overall carriage prevalence of 21% in the first two years of life (76). In agreement with recent data from Kenya, serotype-specific acquisition rates were strongly correlated with serotype carriage prevalence (285). This study also documented a negative correlation between serotype-specific acquisition and clearance rates, which was also seen in the Maela cohort. In the current cohort, having a mother who smoked or other young children in the household was associated with an earlier age at first pneumococcal colonisation. Siblings have been previously recognised as a risk factor for pneumococcal acquisition (107). The presence of smokers in the household has not been consistently identified as a risk factor for infant pneumococcal carriage, although infants in India who were exposed to ≥20 cigarettes per day were at increased risk of colonisation at two months of age (110).

Carriage duration varied by serotype, with serotypes 19F and 23F being carried for the longest periods in infants. For serotypes 14 and 19F, previous carriage of the serotype was associated with significantly increased interval to reacquisition and shorter duration of subsequent carriage of the same serotype. This trend was observed for 23F, although the reduction in carriage duration did not reach significance. These findings suggest that nasopharyngeal exposure to serotypes with immunogenic capsular polysaccharides (14/19F > 23F > 6B) result in an immune response capable of enhancing clearance if reacquisition

occurs. The fact that NT carriage was also associated with delayed reacquisition of NT pneumococci implies that other factors, such as antibody responses to common surface proteins, are also involved in the regulation of pneumococcal nasopharyngeal colonisation. Modelling of data from previous longitudinal carriage studies has demonstrated evidence for both serotype-dependent and independent effects on pneumococcal serotype acquisitions in young children, but not both in the same study population. Carriage of eight serotypes was modelled in Israeli infants aged 12 – 35 months, and a significantly lower risk of acquisition of serotypes 6A, 14, and 23F in those previously colonised was found (120). Serotype-independent protection against reacquisition of four serotypes was shown in Bangladeshi infants aged <1 year (121). In univariate and multivariate models, there was no evidence of serotype-specific protection from colonisation in Maela infants. For several serotypes, previously colonised infants had a significantly greater risk of acquisition of the homologous serotype most likely as a result of intense exposure to pneumococci in the household and community. Colonisation by another serotype in the preceding month reduced the risk of acquisition of 6B, 6C, 19A, and 23F, and previous colonisation by 19F protected against subsequent colonisation by 6C. These observations provide some evidence for the occurrence of intra-species competition and serotype-independent protection against colonisation in children aged <2 years.

Eight serotypes accounted for two-thirds of the pneumococcal isolates from infants. Serogroups 6, 19, 23 and serotype 14 have been the most prevalent pneumococci in the majority of previous infant/childhood carriage studies, regardless of geographical location (73, 76, 110, 114). As expected, the proportion of PCV13 serotype carriage was greater in infants than in their mothers (87, 98, 116). An unexpected finding was the high prevalence of non-typeable pneumococcal carriage. These organisms have limited disease potential, but may act as an important reservoir of antimicrobial resistance genes (39). They are not consistently reported in carriage studies: they may not be identified because of atypical colony morphology or they may be actively excluded because of lack of association with

invasive disease. When included, prevalence has varied from 0.7% of pneumococci in a Kenyan survey of adults and children to 44.5% in Spanish primary school children (98, 140). In the current study, these organisms were carried at least once by 55.1% of infants and 32.0% of mothers. The vast majority of isolates appeared non-encapsulated, rather than being pneumococci of a novel capsular type (data not shown). While it is possible that these organisms were misidentified non-pneumococcal streptococci or the result of poor quality serotyping quality control this is unlikely as all were confirmed by optochin susceptibility and bile solubility, and serotype was rechecked by Quellung. In addition 168 NT pneumococci from this collection have been genotyped by MLST and 96.4% were typed successfully, confirming their identity (134, 286). Further characterisation of a small sample of isolates by microarray-based comparative genomic hybridisation and subsequent sequencing of the *cps* locus, confirmed their pneumococcal identity and demonstrated deletion or disruption of the capsule biosynthesis genes in 17/18 (94.4%) isolates (139).

Antimicrobial resistant pneumococci were frequently carried by infants and their mothers, with PCV13 serotypes predominating. Serotypes 6B, 19F, 19A, and 23F accounted for around two-thirds of all MDR pneumococcal acquisitions, a finding in agreement with previously published data (45). Interestingly, recent antimicrobial consumption was associated with an increased risk of an infant becoming colonised by serotype 19F. It would be expected that introduction of a pneumococcal conjugate vaccine into the community would result in a reduction in carriage and disease caused by drug-resistant pneumococci. However, non-typeable pneumococci were also frequently drug resistant: 80.5% of infant isolates were penicillin non-susceptible and 38.5% were MDR. As previously noted, these frequently carried organisms may represent a significant reservoir of anti-microbial resistance genes for exchange with encapsulated pneumococci (39).

There are some limitations to the study. Firstly, the WHO culture protocol underestimates the prevalence of multiple serotype carriage (see section 4). This may result

in an underestimation of carriage duration. It may also explain the emergence of "replacement" serotypes seen when PCV is used and the predominant carriage serotypes decline (69). Secondly, the sampling strategy employed means that there were only limited data regarding adult pneumococcal carriage prevalence and transmission of serotypes within the household. The carriage prevalence in the mothers (24.0%) was higher than that documented in Kenyan adults (5.3%), similar to Papua New Guinean mothers (~30%), but lower than Gambian adults (>50%) (72, 87, 98). Mothers were selected for inclusion since they were likely to have the greatest contact with the infants and therefore offered a reasonable opportunity to study pneumococcal transmission. However, the large household sizes and the crowded nature of the camp inevitably resulted in the majority of pneumococcal transmission being unobserved. In spite of this a trend in concordance of serotypes between mothers and their infants could be documented, suggesting that transmission was more frequent in the early months of life. The monthly interval between nasopharyngeal swabs will have resulted in the under detection of serotypes carried for short durations. This is particularly relevant to the estimation of carriage prevalence in adults, as median carriage duration was only 31 days (i.e. a serotype detected at a single swabbing point only) in the study mothers. This swabbing interval is also likely to generally overestimate serotype carriage episode durations (285). Although antimicrobial consumption data were collected for each individual child, this was likely to be inaccurate in many cases. Antimicrobial prescriptions from the SMRU clinic and PU-AMI hospital were documented but, as is the case with many parts of SE Asia, antimicrobials may be purchased in Maela without prescription from local pharmacies or markets (287). In many circumstances an antimicrobial agent is included in an over-the-counter mixed medication bag to treat fever (288). Measurement of urinary antimicrobial activity at swabbing visits may have permitted a more accurate assessment of the influence of recent antimicrobial consumption on pneumococcal acquisitions and colonisation (289). However, not all antimicrobials are excreted in the urine and only very recent consumption could be

captured in this way. Camp-level antimicrobial consumption/purchase data would have provided a useful indication of the antimicrobial pressures that colonising pneumococci were exposed to. However, given the nature of the camp and the porosity of its borders, these data were not available.

In conclusion, the characteristics of pneumococcal carriage in mothers and infants in an isolated SE Asian community have been comprehensively documented. These data will inform vaccination strategies in this population. Determination of timing of transmission of pneumococci between mother and infant, and the impact of early colonisation by a serotype on subsequent acquisitions, improves understanding of the complex process of early pneumococcal nasopharyngeal colonisation.

4 Detection of multiple pneumococcal serotype colonisation

4.1. Improved detection of nasopharyngeal co-colonisation by multiple pneumococcal serotypes using latex agglutination or molecular serotyping by microarray

4.1.1. Summary

Background

Identification of *S. pneumoniae* in the nasopharynx is critical for the understanding of transmission, estimates of vaccine efficacy and possible replacement disease. Conventional nasopharyngeal swab culture and serotyping following the WHO protocol is likely to underestimate multiple serotype carriage. In this study, the WHO protocol was compared with methods aimed at improving co-colonisation detection.

Methods

125 NPS from infants in the Maela pneumococcal carriage study, containing ≥1 serotype by WHO culture, were re-cultured in duplicate. A sweep of colonies from one plate culture was serotyped by latex agglutination. DNA extracted from the second plate was analysed by *S. pneumoniae* molecular serotyping microarray.

Results

Multiple serotypes were detected in 11.2% swabs by WHO culture, 43.2% by sweep serotyping, and 48.8% by microarray. Sweep and microarray were more likely to detect multiple serotypes than WHO culture ($P < .001$). Co-colonisation detection rates were similar between microarray and sweep but microarray identified the greatest number of serotypes. A common serogroup-type was identified in 95.2% swabs by all methods.

Conclusions

WHO methodology significantly underestimates multiple serotype carriage compared to these alternative methods. Sweep serotyping is cost-effective and field-deployable but may fail to detect serotypes at low abundance whereas microarray is more costly and technology-dependent but may detect these additional minor carried serotypes.

4.1.2. Introduction

The introduction of pneumococcal conjugate vaccines has resulted in a profound reduction in invasive disease caused by serotypes covered by the vaccines, but increases in the numbers of cases due to non-vaccine serotypes have been observed (193). Conjugate vaccines have also been shown to prevent nasopharyngeal carriage of the vaccine serotypes, although an increase in the carriage of non-vaccine serotypes has also been documented (67). To plan future vaccine strategy there is a need to better understand the dynamics of pneumococcal colonisation of the nasopharynx (a critical early step in the pathogenesis of invasive pneumococcal disease, IPD (46)), particularly in relation to carriage of, and competition between, multiple serotypes. Non-vaccine serotypes carried at low density may become important disease-causing serotypes once vaccine serotypes are removed from, or suppressed within, the nasopharyngeal niche by immunisation (155). Longitudinal studies of pneumococcal colonisation allow detailed exploration of carriage dynamics but standard approaches based on agar plate culture and conventional serotyping techniques may not be optimal for the detection of multiple serotype carriage (70). Published studies have reported multiple colonisation prevalence inconsistently and, when this has been reported, laboratory methods have varied considerably making direct inter-study comparisons difficult. Gratten et al. serotyped up to six colonies from nasal swab culture plates and found multiple serotype carriage in 29.5% of Papua New Guinean children (145). They went on to serotype at least 50 colonies from ten selected nasal swab cultures and concluded that the minor carried serotype accounted for 4 – 27% of the total pneumococcal population. In a study of Tanzanian children, Charalambous and colleagues confirmed that single colony selection was a reasonable approach for determination of serotype/group colonisation prevalence at a population level but that multiple colony selection/serotyping would be necessary to accurately define multiple colonisation prevalence and total serotype diversity (272). Hare et al. concluded that random colony selection was superior to selection by morphological difference for determination of

multiple colonisation Australian Aboriginal children (148). A review of published literature on multiple carriage concluded that, to detect a minor carried serotype, it would be necessary to serotype at least five colonies to have a 95% chance of detecting the serotype if it accounted for 50% of the total pneumococcal population and one would need to examine 299 colonies if the serotype was present at a relative abundance of 1% (147). Given the time and financial implications of the study of multiple serotype colonisation by Quellung serotyping of more than a single colony, several alternative approaches for multiple serotype detection have been described. Researchers in The Gambia have developed a latex agglutination technique where colonies from the primary culture plate are suspended in saline and serotyped by latex agglutination. Using this extremely cost-effective method up to 10.4% of pneumococcal acquisitions were found to be of multiple serotypes in a longitudinal infant cohort study (73). However, there are no published studies comparing this technique with conventional pneumococcal serotyping methodologies for detection of multiple serotype colonisation. Several other methods have demonstrated increased detection of multiple carriage compared to standard culture, including swab enrichment culture followed by either Quellung typing (151) or multiplex PCR (25) from the broth; immunoblotting (150) or multiplex PCR (146) from the primary culture plate; multiplex PCR direct from the nasopharyngeal swab - STGG transport medium specimen (NPS-STGG) (153); *ply*NCR PCR followed by terminal RFLP direct from the swab (154); and microarray based detection and serotyping (29, 142). However, in general these methods are either expensive, time-consuming, limited in number of serotypes detected, or a combination of these factors and therefore are not well suited for use in large scale carriage studies, particularly those carried out in less developed countries where the largest burden of pneumococcal disease resides (2).

4.1.3. Aims and objectives

The objectives for this study were firstly to determine the amount by which the standard WHO culture method for detection of pneumococcal nasopharyngeal carriage underestimates co-colonisation by multiple serotypes and secondly to assess the utility of the two alternative methods, one molecular (for reference laboratory use) and the other culture-based (for field laboratory use), for detection of pneumococcal co-colonisation in an infant carriage study. Micro-array was selected as the reference laboratory method since it had previously been demonstrated to be able to detect extremely low abundance pneumococci of any known serotype (29). The latex agglutination-sweep method developed in The Gambia was selected for the SMRU laboratory since it was cheap, rapid, suited to high-volume studies, technically undemanding, and could theoretically detect all known pneumococcal serotypes (73).

4.1.4. Methods

Nasopharyngeal swabs collected from cohort infants during the Maela pneumococcal carriage study were used in the current study.

4.1.4.1. Detection of pneumococcal colonisation by the WHO culture protocol

Swabs were collected and processed according to the current WHO pneumococcal carriage detection protocol, as described in sections 2.2.4.1 and 2.2.5.1 (70).

4.1.4.2. Detection of pneumococcal colonisation by sweep serotyping or microarray

One hundred and twenty five NPS-STGG specimens known to contain at least one pneumococcal serotype by the WHO culture method were selected from the entire swab collection. Laboratory personnel were blinded to the original WHO culture result.

The specimens were thawed, mixed, and 10 μL aliquots of STGG were re-cultured on to two CNA-blood agar plates. Both plates were incubated overnight at 36 °C in 5% CO_2. Growth was assessed after overnight culture and those with growth only in the

primary streak were re-incubated for a further twenty four hours. If growth was still poor at 48 hours, the specimen was re-cultured using 50 µL aliquots of STGG. The entire growth from each plate was removed using a sterile cotton swab (Medical Wire & Equipment, Corsham, UK) and suspended into aliquots of sterile saline. The first aliquot (0.5 – 2 mL saline, adjusted to make an organism density of 0.5 McFarland) was used for sweep serotyping and genomic DNA was extracted from the second aliquot using a Gram positive bacteria protocol (QIAamp mini kit, Qiagen, Hilden, Germany). Briefly, the organism suspension was centrifuged to pellet the bacteria; this pellet was resuspended in 180 µL of an enzyme lysis solution (20 mg/mL lysozyme; 20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton) and incubated at 37 °C for 30 minutes. Two hundred microlitres of Qiagen buffer AL and 20 µL proteinase K were added and the mixture incubated for 30 minutes at 56 °C followed by 15 minutes at 95 °C. Following these lysis steps, the standard manufacturer's extraction protocol was followed and DNA was eluted into 200 µL Qiagen buffer AE. The DNA extract was stored at -80 °C prior to testing by the Bacterial Microarray Group (Drs Jason Hinds and Kate Gould) at St. George's Hospital, University of London, UK (BµG@S; http://bugs.sgul.ac.uk).

Sweep serotyping by latex agglutination was performed on the saline suspension: the suspension was first tested with antisera pools A-I, followed by all appropriate group, type, and factor antisera (described in section 12.3 & reference (73)). Non-typeable pneumococci were provisionally identified by the latex agglutination reactions described previously.

Molecular serotyping was performed on the DNA extracts using the BµG@S SP-CPSv1.4.0 microarray and the output analysed using a Bayesian hierarchical model as described elsewhere (142). In addition to serotype detection, the array was used to determine the relative serotype proportions in specimens containing multiple serotypes (29).

To confirm a single highly discrepant result, a swab in which sweep serotyping identified two serotypes but microarray detected nine serotypes, intensive study of the NPS-STGG specimen by conventional culture and serotyping was undertaken. To ensure single colonies would be well separated, five 2 μL aliquots of the thawed STGG were re-cultured onto CNA-blood agar plates and incubated overnight as described previously. A total of 300 randomly selected alpha-haemolytic colonies were sub-cultured onto plain blood agar plates (with optochin discs to identify *S. pneumoniae*) and subsequently serotyped by latex agglutination. Finally, sequential multiplex PCR was attempted on the DNA extract from this specimen culture to provide a second confirmatory molecular serotyping result (23).

4.1.4.3. Statistical analysis

Continuous variables were analysed by the Wilcoxon matched-pairs signed-rank test and proportions were compared by the chi-squared test. Comparisons of multiple serotype carriage detection by method were done using McNemar's test. Statistical significance was inferred by two-sided *P*-values of $< .05$.

At the time of this study it was not possible to resolve serotypes 6A and 6C using antisera, therefore these serotypes were considered identical and labelled 6A/C. In a small number of specimens microarray testing identified multiple non-typeable pneumococci, based on variation of the *cps* locus components, however these were considered to be a single serotype when comparing methods.

4.1.5. Results

4.1.5.1. Swabs and study participants

One hundred and twenty five nasopharyngeal swabs from 42 infants, aged between 26 and 516 days, were included. Swabs from up to 12 different sampling time points per infant were examined.

4.1.5.2. Detection of multiple serotype colonisation

Multiple pneumococcal serotypes were identified in 14/125 (11.2%) of the NPS by the WHO culture method. A single serotype was identified in the other 111/125 (88.8%) swabs. The proportion of swabs in which multiple serotype carriage was identified increased using either sweep (54/125; 43.2%) or microarray serotyping (61/125; 48.8%). Sweep serotyping was 3.9 times (95% CI 2.4 – 6.2) and microarray serotyping 4.4 times (95% CI 2.8 – 6.9) more likely to detect multiple pneumococcal serotype colonisation than the WHO culture method ($P < .001$).

Considering all 125 swabs together, microarray identified significantly more pneumococci (222 pneumococci / 47 serotypes) than either sweep serotyping (195 pneumococci / 39 serotypes) or WHO culture (139 pneumococci / 35 serotypes) ($P < .001$) (Figure 15). The number of serotypes identified per swab ranged from one to nine, and is summarised in Table 22.

Figure 15. Distribution of serotypes identified by each detection method

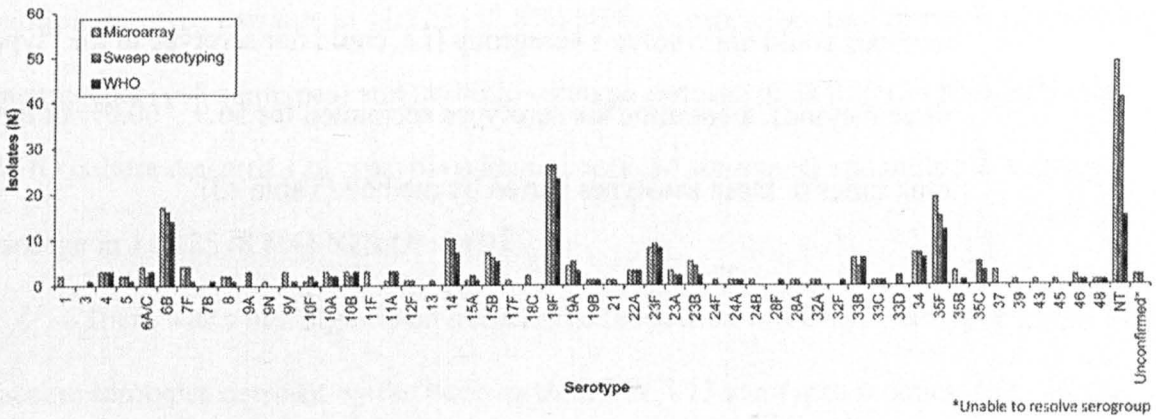


Table 22. Number of pneumococcal serotypes identified per nasopharyngeal swab by each of the three detection methods

Total number of serotypes detected	WHO culture [N (%)]	Sweep serotype [N (%)]	Microarray [N (%)]
1	111 (88.8)	71 (56.8)	64 (51.2)
2	14 (11.2)	41 (32.8)	36 (28.8)
3	0 (0)	10 (8.0)	20 (16.0)
4	0 (0)	3 (2.4)	3 (2.4)
≥5	0 (0)	0 (0)	2 (1.6)

Sweep serotyping detected significantly fewer serotypes per swab than microarray ($P = .04$) although this difference became non-significant ($P = .06$) if a single outlying swab was excluded. In this particular swab, nine serotypes were identified by microarray but only two serotypes by sweep serotyping and by further examination of 300 discrete alpha-haemolytic colonies by conventional culture (246/300 colonies were identified as *S. pneumoniae* and only these were serotyped), although multiplex PCR also indicated the presence of at least four serotypes which included those identified by sweep serotyping plus others detected by microarray. In two instances, the sweep and microarray serotyping methods could not resolve a serogroup (i.e. could not serotype to the "type" level). By all three methods, a common six serotypes accounted for 56.9 – 60.0% of the total but the rank order of these serotypes varied by method (Table 23).

Table 23. Pneumococcal serotypes most frequently identified by each detection method

Serotype	WHO culture [N (%)]	Sweep serotype [N (%)]	Microarray [N (%)]
19F	23 (16.6)	26 (13.3)	26 (11.7)
NT	15 (10.8)	41 (21.0)	49 (22.1)
6B	14 (10.1)	16 (8.2)	17 (7.7)
35F	12 (8.6)	15 (7.7)	19 (8.6)
23F	8 (5.8)	9 (4.6)	8 (3.6)
14	7 (5.0)	10 (5.1)	10 (4.5)
Proportion of total serotypes identified	79/139 (56.9)	117/195 (60.0)	129/222 (58.2)

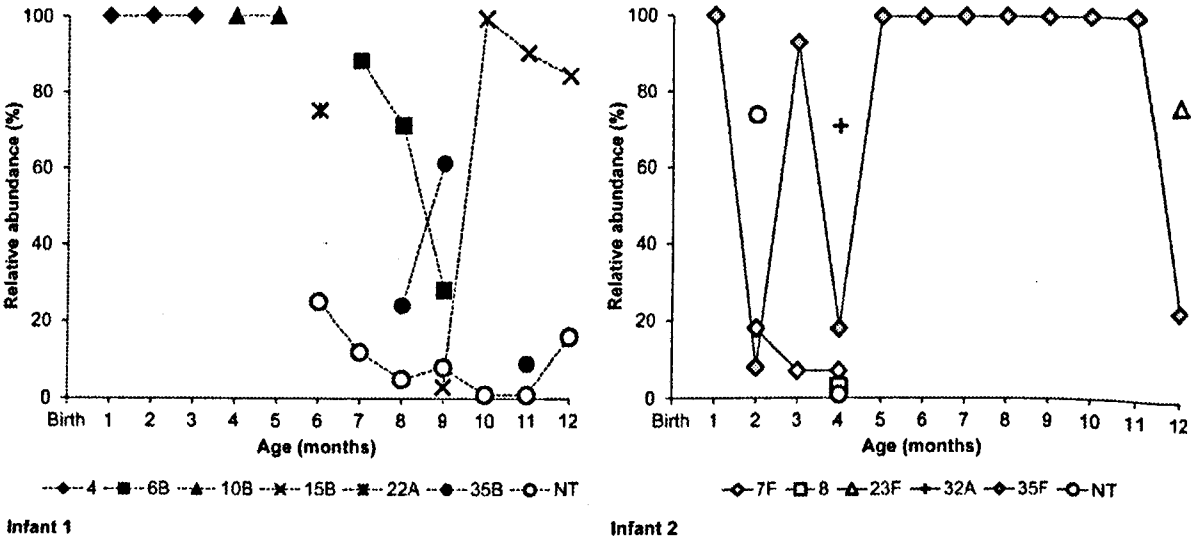
Both sweep and microarray serotyping identified a significantly larger proportion of NT pneumococci compared to WHO culture (22.1% by microarray / 21.0% by sweep vs. 10.8% by WHO, $P = .007$). However, even when these NT pneumococci were excluded from the analyses, sweep or microarray serotyping still identified a significantly greater number of pneumococci and multiple serotype co-colonisation episodes than the WHO culture method. Microarray detected 173 typeable pneumococci (46 serotypes) and multiple serotype carriage in 41/125 (32.8%) NPS; sweep serotyping detected 154 typeable pneumococci (38 serotypes) and multiple serotype carriage in 31/125 (24.8%) NPS; and WHO culture detected 124 typeable pneumococci (34 serotypes) and multiple serotype carriage in 11/125 (8.8%) NPS ($P < .001$).

There was a non-significant trend in the proportion of PCV13 serotypes versus non-vaccine serotypes detected by the three methods: PCV13 serotypes accounted for 46.8% (WHO culture), 39.5% (sweep serotyping), and 38.3% (microarray serotyping) of all of the serotypes detected ($P = .25$). Considering only the microarray data, in swabs where a single serotype was identified (i.e. completely "dominant"), the serotype was a PCV13 type in 35/64 (54.7%) but in swabs where multiple serotypes were detected, the "dominant" serotype was a PCV13 type in only 21/61 (34.4%, $P = .02$). The ability of the

microarray to determine the relative abundance of each serotype allowed detailed exploration of the temporal flux of relative serotype levels in two representative infants, in whom a total of 24 swabs were examined (Figure 16).

Figure 16. Dynamics of pneumococcal carriage over the first year of life in two infants

The figure summarises microarray data of serotypes detected and their relative abundance.



4.1.5.3. Serotype result agreement between detection methods

Overall, at least one common serotype was found by all three detection methods in 109/125 specimens (87.2%; 95% CI 81.1 – 93.1%). Relaxing agreement to the serogroup-type (SGT) level, there was at least one common SGT identified by all three methods in 119/125 specimens (95.2%; 95% CI 91.4 – 99.0%) and SGT agreement between two of the three methods in the remaining six specimens.

In 44 specimens a single pneumococcal serotype was identified by all three detection methods. In 42/44 (95.5%; 95% CI 89.0 – 100%) all three methods identified the same serotype. In one swab WHO and microarray both identified a type 9V pneumococcus but sweep serotyping determined the serotype to be 9A. In the other non-congruent

specimen, both WHO culture and sweep serotyping identified a non-typeable pneumococcus but the microarray detected type 6B capsule genes.

4.1.5.4. Limits of detection of sweep serotyping and microarray

In order to assess the limits of the sweep serotyping assay, serial dilutions of overnight cultures of *S. pneumoniae* (six clinical isolates; serotypes 6A, 6B, 14, 19F, 19A, and 23F) were made in normal saline and these were serotyped using latex antisera as described previously. Organism density in the saline suspensions was determined by the plate count technique (290). Reproducible serotype results could be obtained at organism densities of $10^7 - 10^8$ CFU/mL (i.e. 0.5 McFarland). In vitro mixtures of these suspensions confirmed that, at an overall organism density of 0.5 McFarland (visual), serotypes present at ≥25% of the total pneumococcal population could reliably be detected by latex agglutination (data not shown).

In the 61 swabs in which multiple serotypes were detected by microarray, the relative proportion of the minority serotype (defined as the least abundant serotype detected) ranged from 1% to 46% (median 7%, IQR 3 – 14%) (Table 24). Microarray serotyping detected additional serotypes compared to sweep serotyping in 38 specimens, in 30/38 (78.9%) the predominant additional serotype detected was NT and in 21/38 (55.3%) the only additional serotype was NT. In the 17/38 specimens in which microarray detected additional typeable pneumococci, 9/17 (53.0%) serotypes were present at <10%, 5/17 (29.4%) were present at 10 - 19%, and only 3/17 (17.6%) were present at ≥20% of the total pneumococcal population.

Table 24. Relative proportion of minority serotypes detected by microarray compared with the total number of pneumococcal serotypes detected

Relative proportion of minority serotype	Total number of serotypes detected					Total
	2	3	4	5	9	
<10%	12	15	3	1	1	32
10-19%	11	5	0	0	0	16
20-29%	6	0	0	0	0	6
30-39%	3	0	0	0	0	3
40-49%	4	0	0	0	0	4
Total	36	20	3	1	1	61

4.1.6. Discussion

This study has demonstrated that culture and serotyping of nasopharyngeal swab specimens using the current WHO protocol significantly underestimates the frequency of multiple pneumococcal serotype carriage. Sweep serotyping identified 1.4 times, and microarray 1.6 times, as many pneumococci compared to this standard culture method. These alternative methods also revealed greater diversity of carriage serotypes, reflected by the greater proportion of non-vaccine serotypes identified by each method. As expected, microarray identified the largest number of carried serotypes and was able to detected minor serotypes present at very low relative abundance. The microarray data confirmed the previously described finding that the minority serotype in a multiple carriage episode is present at low abundance: we documented a median relative abundance of 7% (range 1 – 46%) for the minority serotype, compared with 4 – 27% found in the Papua New Guinea study using Quellung typing of multiple colonies (145). The ability of microarray and sweep serotyping to confidently document the frequency of non-typeable pneumococcal carriage is also useful since these organisms are suspected of being an important reservoir of antimicrobial resistance although they are, as yet, not an important cause of invasive disease (39). Identification of non-typeable pneumococci can be problematic given the phenotypic and genetic similarities to closely related streptococcal species such as *S. mitis*

and *S. pseudopneumoniae.* However, characterisation of 168 NT isolates from the Maela study population by MLST concluded that 162/168 (96%) could be assigned a sequence type and only six isolates were found to be non-pneumococcal streptococci by eMLSA analysis (286). This result gives confidence to the finding of frequent NT pneumococcal detections in the current evaluation.

The agreement between the three methods, in terms of detection of a common serotype, was excellent with at least one common SGT identified in 95.2% of swabs. The two discrepancies observed in 44 swabs in which a single serotype was identified by all methods were minor: a factor discrepancy (9A versus 9V) between methods in one and a potentially non-expressed capsule in the other (microarray identified 6B capsule genes but both WHO and sweep found a non-typeable pneumococcus). A single result was unsatisfactory: the swab in which microarray identified nine serotypes but sweep serotyping identified only two, and WHO culture one, serotypes. This specimen was re-cultured and 246 discrete pneumococcal colonies individually serotyped by latex agglutination: the sweep serotyping result was confirmed by this approach and additional serotypes were not encountered. In spite of this, the microarray result may well be correct and is supported to some extent by multiplex PCR results. It is likely that to detect several very low abundance serotypes it would be necessary to serotype even more than the 300 colonies that was attempted here. Furthermore, the presence of conserved capsule polysaccharide genes in other non-alpha-haemolytic or optochin resistant alpha-haemolytic *Streptococcus* spp. in the primary plate DNA extract may have resulted in signal on the array, possibly considered a false positive but reflecting the total gene pool and capacity for capsule polysaccharide production in the sample (152).

The WHO culture method will detect the predominant serotype but statistically cannot be expected to detect low abundant serotypes (147). Increasing the number of colonies serotyped by random selection from the primary culture plate has been shown to improve detection of multiple colonisation (148), but the cost and time required for the

additional serotyping renders this difficult to sustain in the setting of a large carriage study. These factors also limit the utility of immunoblotting or broth enrichment methods in such studies (150, 151).

Sweep serotyping is cheap, quick, technically straightforward and was not significantly inferior to the microarray method in detecting multiple carriage episodes, although it was not efficient at detecting pneumococcal serotypes present at very low abundance (<25% of the total). If this can be improved sweep serotyping may ultimately become a gold-standard method for detection, but not relative quantitation, of multiple serotypes in nasopharyngeal samples.

Molecular techniques, especially when applied directly to the swab, reduce the cost and time of multiple serotype detection compared to Quellung typing but currently multiplex PCR can only detect a limited number of serotypes (29 in the original multiplex (23)) and may therefore miss serotypes ultimately important for replacement disease after vaccination (25, 46). The significant advantages of the microarray method used in the current study are the ability to detect all known pneumococcal serotypes and estimate their relative abundance in a mixed population. In the setting of a longitudinal carriage study, these additional data will permit detailed observations of the interactions between pneumococcal serotypes, and indeed between pneumococci and other bacterial or viral species, over time.

Both sweep and microarray serotyping enable researchers to better interrogate nasopharyngeal swab specimens with regard to detection of multiple pneumococcal serotype carriage. In the current study microarray was more sensitive than sweep serotyping and provided useful data regarding relative serotype abundance. However, microarray-based molecular serotyping is currently restricted to a small number of research laboratories and the cost-per-test is relatively high, although additional simultaneous analyses can be conducted. Sweep serotyping is extremely cost effective (10μL neat pneumococcal antiserum is used to produce 6mL of latex reagent) and can be performed in

any microbiology laboratory capable of serotyping *S. pneumoniae* by Quellung, which makes it a highly useful technique for pneumococcal carriage studies, particularly in resource-poor settings.

4.2. Further evaluations of latex sweep serotyping for detection of multiple pneumococcal serotype colonisation

4.2.1. Summary

Background

Standard culture of nasopharyngeal swabs using WHO methodology to detect pneumococcal colonisation underestimates the prevalence of multiple serotype colonisation. The impact of this on pneumococcal serotype distributions and colonisation dynamics in infancy are not clear.

Methods

To further evaluate the utility of latex sweep serotyping, 8,736 NPS collected from 364 infants taking part in the Maela longitudinal infant pneumonia cohort study were cultured and serotyped using the latex sweep method. 1,107 of these swabs had previously been cultured using the standard WHO methodology. To assess the impact of culture/serotyping methodology on estimation of serotype colonisation dynamics, a retrospective case-control study was done comparing colonisation results on 100 matched pairs of infants. Swabs from each infant were cultured either by WHO or latex sweep method.

Results

In 1,107 swabs cultured by both WHO and latex sweep methods, latex sweep serotyping was three times more likely to detect colonisation with multiple pneumococcal serotypes, excluding NT pneumococci, than WHO culture (9.3% vs. 3.1% of NPS; $P < .001$). At least one common serotype was identified in 91.2% of swabs from which pneumococci were detected by both methods. Agreement between methods improved with increasing colonisation density ($P = .03$). Estimates of age at first pneumococcal acquisition and duration of colonisation were not affected by culture/serotyping method. However, a greater number of serotype carriage episodes were detected in infants cultured by latex

sweep ($P = .03$). The overall rate of NVT pneumococcal acquisition was also greater in infants cultured by latex sweep ($P = .04$).

Conclusions

Latex sweep serotyping was feasible to perform on a large specimen collection. As expected, detection of multiple serotype colonisation was significantly improved compared with WHO methodology. Further work to improve latex sweep serotype detection at low colonisation densities is required.

4.2.2. Introduction

The previous evaluation (section 4.1) demonstrated that standard WHO culture and serotyping was an inadequate tool for detecting and characterising multiple serotype colonisation. This finding raised questions regarding studies of pneumococcal colonisation dynamics:

- Do methodological differences (WHO culture/serotyping vs. culture plus latex sweep serotyping) significantly influence reported serotype distributions?
- Is there a methodological effect on estimates of acquisition rates and carriage duration?

4.2.3. Aims and objectives

The objectives of this study were to perform culture plus latex sweep serotyping on nasopharyngeal swabs collected from cohort infants and to subsequently compare pneumococcal colonisation results both within infants (WHO vs. sweep method in "routine" follow-up group infants) and between groups of infants ("immunology" (WHO culture) vs. "routine" (sweep method) follow-up groups).

4.2.4. Methods

4.2.4.1. Specimen selection and processing

On completion of the Maela pneumonia cohort study follow-up, the specimen database was reviewed and all nasopharyngeal swabs from "routine" follow-up infants with a complete set of 24 monthly specimens were cultured and latex sweep serotyping done as described in section 4.1.4.2. A proportion of these NPS specimens had previously been cultured according to the WHO protocol as described in section 2.2.5.1. The laboratory staff that performed the latex sweep serotyping were blinded to the original WHO culture results.

Colonisation density was assessed by estimating the number of pneumococcal colonies in each of the four agar plate quadrants: 1+ (growth in quadrant 1 (inoculation area/primary streak) but <10 colonies in quadrant 2), 2+ (>10 colonies in quadrant 2 but <10 colonies in quadrant 3), 3+ (>10 colonies in quadrant 3 but <10 colonies in quadrant 4), or 4+ (>10 colonies in quadrant 4).

To compare pneumococcal acquisition rates and carriage durations by culture/serotype method, all "immunology" follow-up group infants with a complete 24 monthly NPS specimen set were matched 1:1 by gender and month of birth with a "routine" follow-up group infant using the "vmatch" command in Stata/IC 12.1 (StataCorp, College Station TX, USA).

4.2.4.2. Definitions of carriage

The definitions of serotype acquisition, clearance, and duration employed were those used in the study of pneumococcal colonisation mother-infant pairs and described in section 3.1.4.3.

4.2.4.3. Data analysis

Comparisons of serotype detections by WHO culture or sweep serotyping were made by McNemar's test. Continuous numerical variables were summarised by means and groups were compared by t-test. Serotype acquisition rates and carriage durations were calculated by survival analysis techniques, as described in section 3.1.4.4. Groups were compared using the log-rank test.

As previously described, serotypes 15B and 15C were considered to be a single serotype (15B/C). Serotypes 6A and 6C were also combined (serotype 6A/C), since, at the time of the study, latex sweep serotyping could not distinguish between the two. Non-typeable pneumococci were excluded from these comparisons, since culture and serotyping by latex sweep has no confirmatory test of pneumococcal identity except the presence of capsule.

4.2.5. Results

4.2.5.1. "Routine" follow-up group infants

Three hundred and sixty four infants from the "routine" follow-up group had a complete 24 month NPS specimen set. A total of 8,736 swabs were cultured and latex sweep serotyping done. Typeable pneumococci were identified in 65.3% (5,699/8,736) of specimens. Multiple pneumococcal serotypes were found in 734 NPS specimens (12.9% of specimens from which pneumococci were cultured). In swabs with more than one serotype, the median number of serotypes detected was two and the maximum was four.

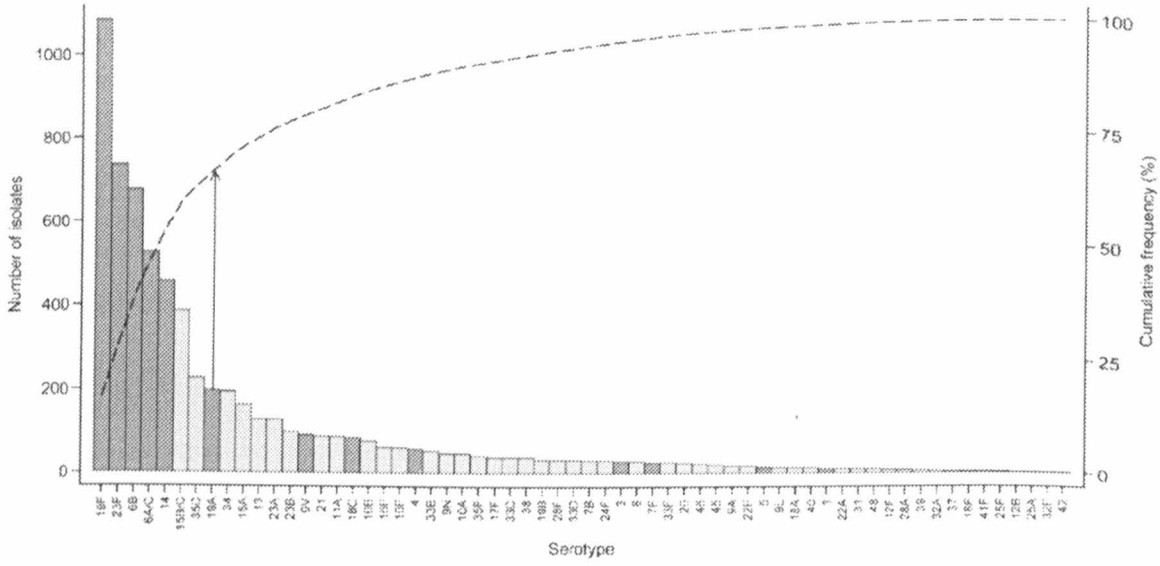
4.2.5.1.1. Serotype distribution

A total of 62 serotypes were identified and eight of these (19F, 23F, 6B, 6A/C, 14, 15B/C, 35C, 19A) accounted for two-thirds of all pneumococci detected (Figure 17). Almost two thirds (61.8%; 3,999/6,471) of the pneumococci detected were PCV13 serotypes and 42.4% (3,704/8,736) NPS contained at least one PCV13 serotype.

The ten serotypes most prevalent serotypes in these infants were identical to those in the 234 infants from the "immunology" follow-up group (excluding NT pneumococci; section 3.1.5.3 and Figure 13), although the rank order varied.

Figure 17. Infant carriage pneumococcal serotype distribution by latex sweep serotyping

Results of culture of 8,736 swabs from 364 infants.



4.2.5.1.2. Agreement with WHO culture within specimens

One thousand one hundred and seven (12.7%) of the NPS specimens from these 364 infants had previously been cultured using the WHO protocol. At least one typeable pneumococcus was identified in 759 (68.6%) and 708 (64.0%) NPS specimens by WHO culture and sweep method, respectively ($P < .001$). Almost ten percent (9.3%, 103/1,107) of specimens contained multiple serotypes by sweep method compared with 3.1% (34/1,107) by WHO method ($P < .001$). However, there were no differences in the proportion of specimens containing at least one PCV13 serotype or NVT pneumococcus by

method (PCV13: 42.7% (sweep) vs. 44.0% (WHO), $P = .1$; NVT: 25.3% (sweep) vs. 26.3% (WHO), $P = .2$).

In swabs containing at least one typeable pneumococcus by the WHO method, an identical serotype was identified in 85.0% (645/759) by the sweep method. Relaxed to agreement at the serogroup/type level, this proportion increased only slightly to 86.4% (656/759). Where both methods identified at least a single typeable pneumococcus, agreement was 91.2% (645/707; serotype level) and 92.8% (565/707; serogroup/type level), perhaps indicating that low density pneumococcal colonisation was missed by the latex sweep method. Serotype agreement between methods was lowest in specimens when there was light colonisation (defined as 1+) on the WHO culture plate (Table 25). Although not statistically significant at the serotype level, the likelihood of agreement at serogroup/type level was significantly higher if colonisation density was 2+ or greater (OR 1.65, 95% CI 1.06 – 2.57, $P = .03$).

Table 25. Relationship between colonisation density and agreement between WHO culture and latex sweep result

Colonisation density (WHO culture plate)	Serotype agreement N (%)	Total
1+	311 (80.4)	387
2+	170 (89.5)	190
3+	96 (92.3)	104
4+	68 (87.2)	78
Total	648 (85.0)	759

4.2.5.2. Comparison of pneumococcal colonisation dynamics: infant swabs cultured by WHO or latex sweep methods

One hundred "immunology" follow-up group infants whose NPS had been cultured by WHO method were matched 1:1 with "routine" follow-up group infants whose swabs

were processed by the latex sweep method. All infants contributed 24 monthly swabs and all were colonised by pneumococci at least once.

In both groups, the median age at first pneumococcal acquisition was 46 days (P = .6; Figure 18). Considering individual serotype acquisition rates, there were no significant differences in rates of acquisition of commonly carried PCV13 serotypes. However, non-vaccine serotypes were acquired faster in infants whose swabs were processed by the latex sweep method (Table 26). Durations of first carriage episodes, without stratification by serotype, were similar in both groups ("immunology"/WHO: median 90 days (IQR 31 – 151); "routine"/sweep: 62 days (IQR 31 – 121); P = .2; Figure 19).

Infants with swabs processed using the latex sweep method had a greater number of pneumococcal serotype carriage episodes than those whose swabs were cultured using WHO methodology (mean 8.4 vs. 7.5, P = .03). There were no differences in the number of PCV13 or NVT serotype carriage episodes between the groups (PCV13 serotypes accounted for 51.4% ("immunology"/WHO) and 54.0% ("routine"/sweep) carriage episodes, P = .3).

Figure 18. Age at first pneumococcal acquisition, by NPS culture/serotyping method

100 infants were included in each group.

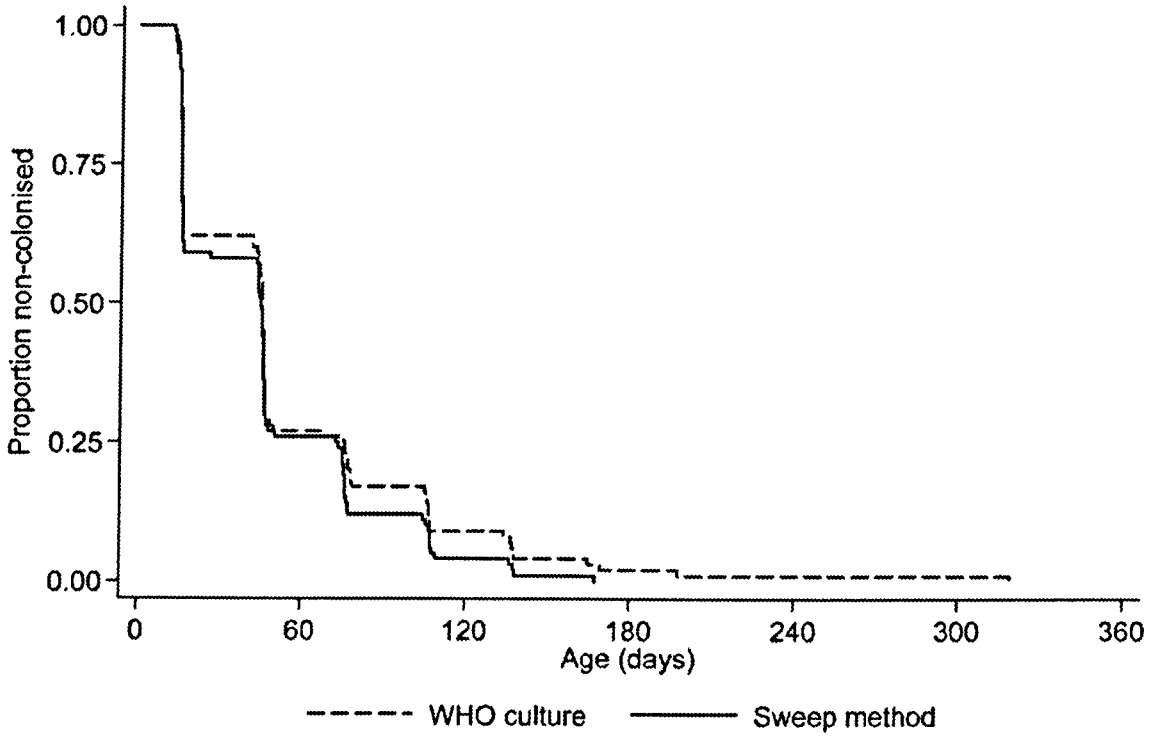


Table 26. Individual pneumococcal serotype acquisition rates, by culture/serotyping method

100 infants were included in each group.

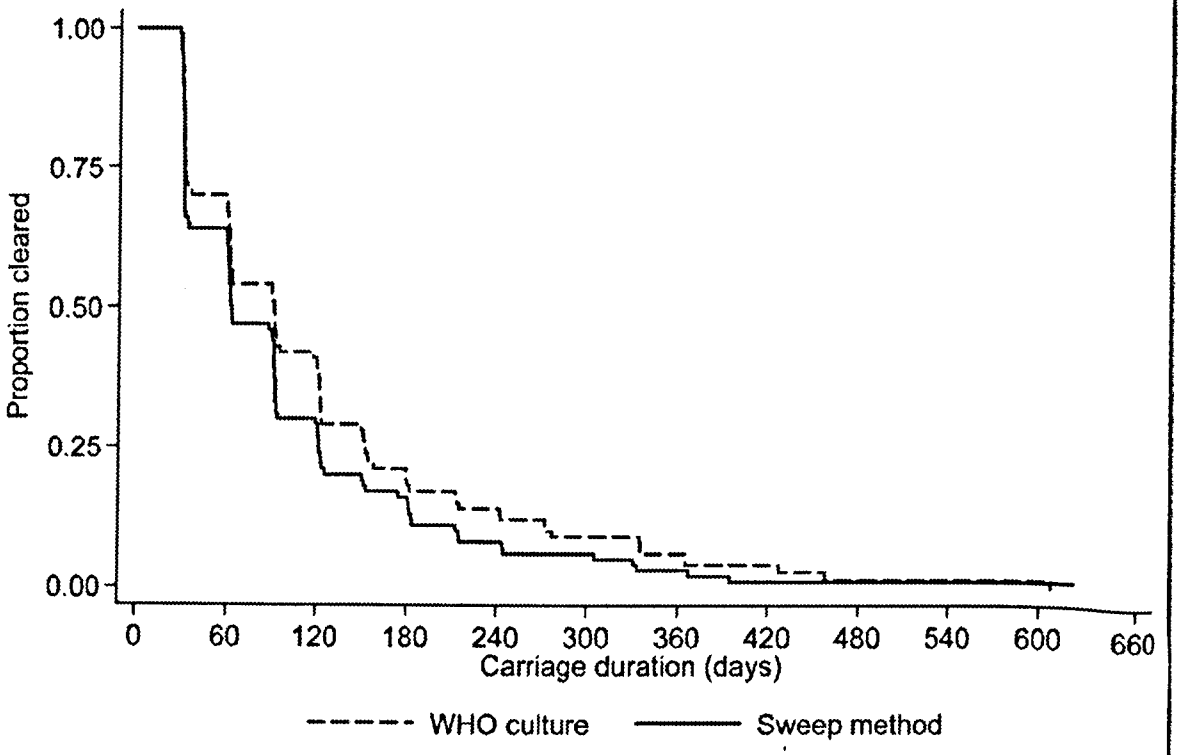
Serotype	Swabs cultured: WHO method			Swabs cultured: sweep method			*P*
	N	Acq rate[a]	95% CI	N	Acq rate[a]	95% CI	
19F	56	0.0012	(0.0009 - 0.0016)	59	0.0012	(0.0010 - 0.0016)	.9
23F	51	0.0010	(0.0008 - 0.0013)	44	0.0008	(0.0006 - 0.0011)	.3
6B	49	0.0009	(0.0007 - 0.0012)	50	0.0009	(0.0007 - 0.0012)	.9
6A/C	47	0.0008	(0.0006 - 0.0011)	48	0.0009	(0.0007 - 0.0012)	.6
15B/C	36	0.0006	(0.0004 - 0.0008)	51	0.0009	(0.0007 - 0.0012)	.03
14	35	0.0006	(0.0004 - 0.0008)	37	0.0006	(0.0004 - 0.0009)	.8
19A	25	0.0004	(0.0003 - 0.0006)	32	0.0005	(0.0004 - 0.0007)	.3
34	22	0.0003	(0.0002 - 0.0005)	32	0.0005	(0.0004 - 0.0008)	.1
15A	15	0.0002	(0.0001 - 0.0004)	20	0.0003	(0.0002 - 0.0005)	.4
35C	12	0.0002	(0.0001 - 0.0003)	29	0.0005	(0.0003 - 0.0007)	.002
PCV13	100	0.0058	(0.0048 - 0.0071)	100	0.0070	(0.0057 - 0.0085)	.2
NVT[b]	92	0.0047	(0.0038 - 0.0057)	98	0.0071	(0.0058 - 0.0086)	.04
All[b]	100	0.0150	(0.0123 - 0.0183)	100	0.0165	(0.0135 - 0.0200)	.6

[a] Acquisition rate per day

[b] Non-typeable pneumococcal colonisation excluded

Figure 19. Duration of first pneumococcal carriage episode, by culture/serotyping method

100 infants were included in each group.



4.2.6. Discussion

This study confirms the preliminary finding that the latex sweep serotyping method is significantly more sensitive than WHO culture followed by serotyping of morphologically distinct colonies for detection of multiple pneumococcal serotype colonisation. It also demonstrates the feasibility of processing large numbers of NPS specimens using the latex sweep method: over 8,000 swabs were turned around in less than 12 months. Alternative methods developed to improve multiple serotype colonisation are currently unlikely to be able to manage such numbers, especially when factoring in the costs of molecular assays or those phenotypic assays requiring significant volumes of pneumococcal antisera (150, 153, 154). Increased throughput did not negatively impact on serotype agreement between two methods: a common serotype was found in 92.8% swabs.

In the initial evaluation of serotyping methods, which included microarray, a common serogroup/type was identified in 95.2% specimens (291). This is an important finding, especially since recent work has determined that molecular serotyping directly from broth enrichment cultures may identify *cps* locus genes present in non-pneumococcal streptococci resulting in false positive "pneumococcal" serotype detections (152).

Given the high prevalence of carriage of non-typeable pneumococci, improved confirmation of colonisation by these organisms would be helpful (39). Although non-specific weak agglutination by latex can presumptively identify these organisms, without a confirmatory test of pneumococcal identity their carriage prevalence may be over-estimated by the latex sweep method (unpublished data from the PneuCarriage study; personal communication from Catherine Satzke). The finding that the latex sweep method was significantly less sensitive at serotype detection from swabs with lower pneumococcal density is also a problem and further work to improve this is required, especially for the technique to be successfully employed in situations where low colonisation density may be anticipated (156, 292). This is relevant since studies of colonisation in immunised infants and also adults are of considerable importance in monitoring for changing in serotype carriage prevalence following introduction of conjugate vaccines (69). It may also explain why the overall prevalence of multiple serotype colonisation reported here (12.9% by sweep in the "routine" cohort infants) was lower than in some other studies, for example Papua New Guinea where Gratten et al. identified multiple serotypes in 29.5% of children carrying pneumococci (145). However, the three-fold increase in multiple serotype detection (9.3% vs. 3.1%) when comparing sweep results to WHO culture/serotyping in the same specimen selection from Maela infants represents a reasonably impressive increase in detection. In the current study, WHO culture equated to serotyping all morphologically discrete pneumococci. Comparing this strategy to selection of four random colonies for characterisation, Hare et al. identified an additional 3% (14% vs. 17%) of NPS specimens containing multiple pneumococcal serotypes in Australian Aboriginal

children: put another way, random colony selection resulted in a 1.2-fold increase in multiple colonisation detection over the WHO approach (148).

Infant serotype distribution was not affected by culture/serotyping methodology in this study. Excluding non-typeable pneumococci, the top ten ranking serotypes in infant swabs processed by WHO method or latex sweep were identical, with a small number of serotypes accounting for the majority of pneumococci identified. This finding agrees with the work of Charalambous and colleagues, who demonstrated that population-level characteristics of colonising pneumococci could be adequately described by characterisation of a single colony per specimen (272). Age at first pneumococcal acquisition was identical, and durations of first pneumococcal carriage were similar, in infants whose swabs were processed by either method. Estimates for NVT acquisition rates were higher (both overall and for serotypes 15B/C and 35C individually) in infant whose swabs were processed by latex sweep. These infants were also determined to have a greater number of discrete serotype colonisation episodes compare to infants whose swabs were cultured by the WHO method. Sweep serotyping was not significantly more likely detect NVT pneumococcal colonisation overall. However, a limitation of this study was that differences observed in colonisation dynamics in the case-control component may reflect unaccounted for, and potentially important, environmental factors (51). Infants were matched by gender and month of birth, but household differences were not measured.

In conclusion, this study has demonstrated accuracy and feasibility of latex sweep serotyping for detection of pneumococcal colonisation in longitudinal infant studies. Further work to improve detection of colonisation by non-typeable organisms and pneumococci at low density is required.

5 Immunological aspects of pneumococcal colonisation in young children

5.1. Serum antibody responses to pneumococcal capsular polysaccharides in children under two years

5.1.1. Summary

Background

Antibodies against the pneumococcal capsule may protect against nasopharyngeal colonisation. However, detailed kinetics of anti-capsular IgG antibody development in relation to nasopharyngeal acquisitions over the first two years of life are poorly defined.

Methods

Monthly sera and nasopharyngeal swabs were collected from 36 infants from 1 – 24 months of age. Swabs were cultured to detect pneumococcal colonisation and IgG to dominant serotypes (6B, 14, 19F, 19A, and 23F) were quantified.

Results

Pneumococcal colonisation occurred early in the cohort. Anti-capsular IgG declined until six months and failed to protect against colonisation. Increases in homologous anti-capsular IgG were observed for all 19A acquisitions but only to a proportion of others (from 23F - 29.2% to 14 - 76.9%). Older age at acquisition was associated with a greater increase in homologous anti-capsular IgG ($\beta = 0.05$; 95% CI 0.03 – 0.07; $P < .001$). Anti-capsular IgG fold-change between six and 24 months to serotype 14 was greater in those infants who had been colonised compared with the non-colonised group (3.64 vs. 0.41, $P < .001$).

Conclusions

The relationship between serum IgG and the prevention of, or response to, pneumococcal nasopharyngeal colonisation remains complex. Mechanisms other than serum anti-capsular IgG are likely to have a role but are currently poorly understood.

5.1.2. Introduction

The immune response to pneumococcal nasopharyngeal colonisation is complex and remains incompletely understood. Murine models have suggested a relatively limited role for antibody in the clearance of nasopharyngeal colonisation and that a CD-4^+ T-cell pathway mediated by IL-17A is the dominant mechanism involved in immunity to pneumococcal colonisation (177, 178). However, colonisation in adult humans does result in a rise in serum serotype-specific anti-capsular polysaccharide IgG (183, 184), although these antibodies may not protect against colonisation (185). Pneumococcal conjugate vaccine studies in young children have shown that serum anti-capsular IgG protects against nasopharyngeal acquisition by, and may reduce colonisation density of, pneumococcal serotypes covered by the vaccine (67, 120, 156, 187). It has been suggested that a serum anti-capsular IgG concentration of $\geq$0.35 μg/mL is protective against invasive disease of the homologous serotype (293, 294). Whilst mucosal IgA antibodies are produced in response to pneumococcal colonisation and infection, correlates of protection for either colonisation or invasive disease are not yet known (295-297).

Previous studies of pneumococcal colonisation and immune responses in infants and young children have demonstrated the generally limited development of serotype-specific serum anti-capsular antibodies in the absence of PCV immunisation, although these studies included relatively infrequent sampling points which restricted potential for study of the immune response to individual pneumococcal serotype acquisitions (188, 190). Also, failure to pre-adsorb test sera with both pneumococcal cell wall polysaccharide and type 22F capsular polysaccharide has been demonstrated to result in sub-optimal quantitation of serotype-specific anti-capsular antibody responses in older generations of antibody detection assays (298, 299).

5.1.3. Aims and objectives

The aims of the present study were to examine: (i) the effect of maternally derived serotype specific anti-capsular IgG antibodies on risk and timing of nasopharyngeal colonisation by the homologous serotype in infancy; (ii) the development of serum serotype-specific anti-capsular IgG in relation to nasopharyngeal acquisitions of pneumococci in the first two years of life.

5.1.4. Methods

5.1.4.1. Study site and population

Thirty six infants from the "immunology" follow-up group of the Maela cohort, with complete sets of both nasopharyngeal swabs and serum specimens, were selected for further analysis.

5.1.4.2. Pneumococcal nasopharyngeal colonisation

Nasopharyngeal swabs were taken at scheduled monthly visits from one to 24 months of age (section 2.2.3). Swabs were collected and processed according the standard WHO protocol, as described in sections 2.2.4.1 and 2.2.5.1 (70, 291). All pneumococcal isolates were serotyped by latex agglutination with Quellung confirmation of equivocal results (273).

5.1.4.3. Determination of pneumococcal capsular antibody concentrations

At each monthly nasopharyngeal sampling point a venous blood specimen was collected into a 2 mL plain tube (Teklab, Sacriston, UK) and an aliquot of serum was stored at -80°C for subsequent antibody analyses. Serum IgG antibodies to capsular polysaccharides 6B, 14, 19F, 19A, and 23F were measured (subsequently referred to as "target" serotypes). These serotypes were selected since they are included in the 13-valent conjugate vaccine (PCV13) and were frequently carried by infants in the cohort (accounting for 1,573/3,363 (46.8%) of all pneumococci cultured from nasopharyngeal

swabs) (300). Antibody concentrations were determined by ELISA, after adsorption with both 22F polysaccharide and cell-wall polysaccharide, at the WHO reference laboratory for pneumococcal serology in the Institute of Child Health, London, UK (298). The assay limit of detection was 0.15 μg/mL and all results below this were reported as 0.075 μg/mL.

5.1.4.4. Statistical analysis

Antibody concentrations and fold-changes in antibody levels were normalised by log-transformation prior to analysis. Geometric mean concentrations and their 95% confidence intervals were calculated and comparisons between groups were made using t-tests or ANOVA as appropriate. Pearson's correlation coefficients were used to examine correlations between antibody concentrations to the five capsular polysaccharides. The relationship between age at carriage acquisition and anti-capsular antibody response was assessed by linear regression. For this analysis, pneumococcal serotype acquisition was defined as the first ever identification of a serotype from a nasopharyngeal swab culture or when a serotype was re-cultured following clearance as defined by the absence of the serotype from two consecutive swabs. Assessments of the effect of maternally derived serum anti-capsular IgG on risk and timing of homologous serotype acquisition in infants was made by logistic regression and survival analysis, respectively.

5.1.5. Results

Eight hundred and sixty four serum specimens were analysed. The demographic and pneumococcal colonisation characteristics of this sub-group of 36 infants were not significantly different to overall infant cohort (described in chapter 3).

5.1.5.1. Pneumococcal colonisation

The median age at first pneumococcal acquisition was 46 days and all 36 infants had been colonised by the seventh month visit (Figure 20). Over the first two year of life, pneumococci were detected in a median of 20 swabs per infant (IQR 16 – 21; range 8 –

24). Infants were colonised by a median of seven individual pneumococcal serotypes (IQR 6 - 8; range 2 - 11). There were 106 discrete nasopharyngeal acquisitions of the five target serotypes: almost all infants (35/36; 97.2%) carried at least one of the target serotypes and two-thirds (23/36; 63.8%) carried two or more (cumulative acquisitions of target serotypes are summarised in Figure 20). Serotype 19A was acquired later than the other serotypes: median age at first positive swab was 17 months (IQR 11 – 19) versus 9 months (IQR 5 – 14) for the other serotypes combined ($P = .03$; Figure 21).

Figure 20. Cumulative proportion of infants colonised by pneumococci

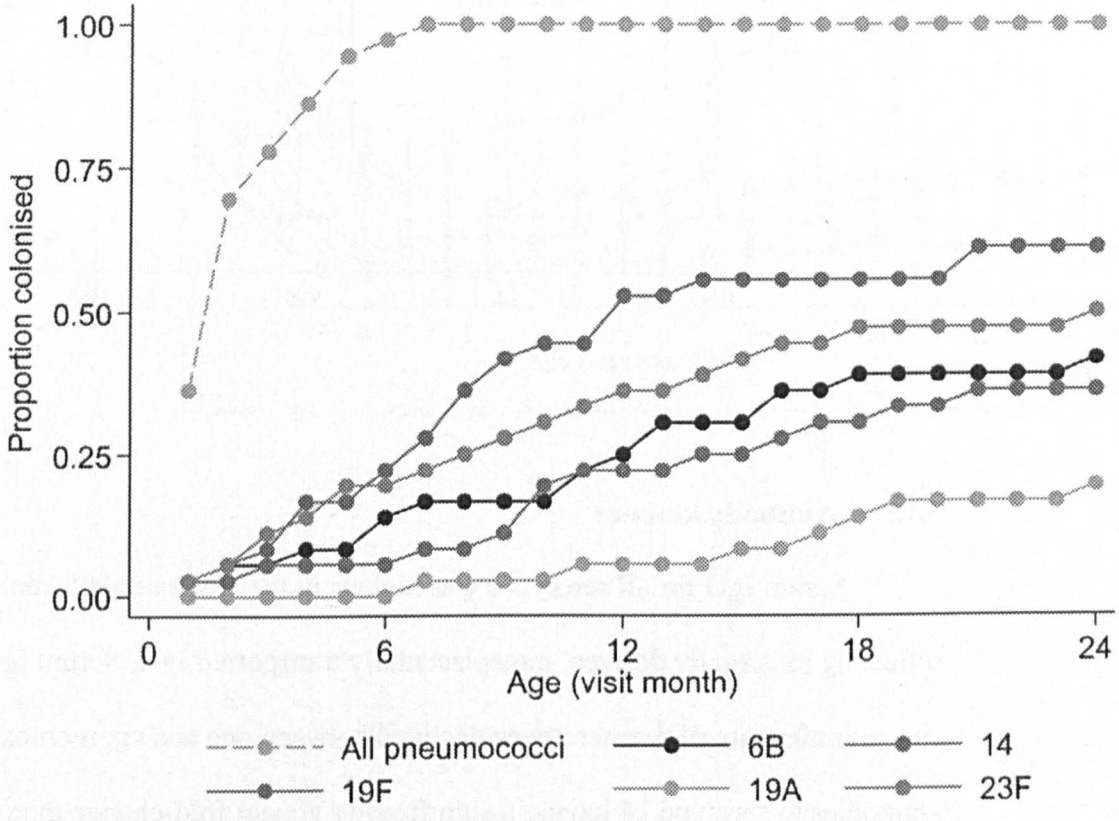
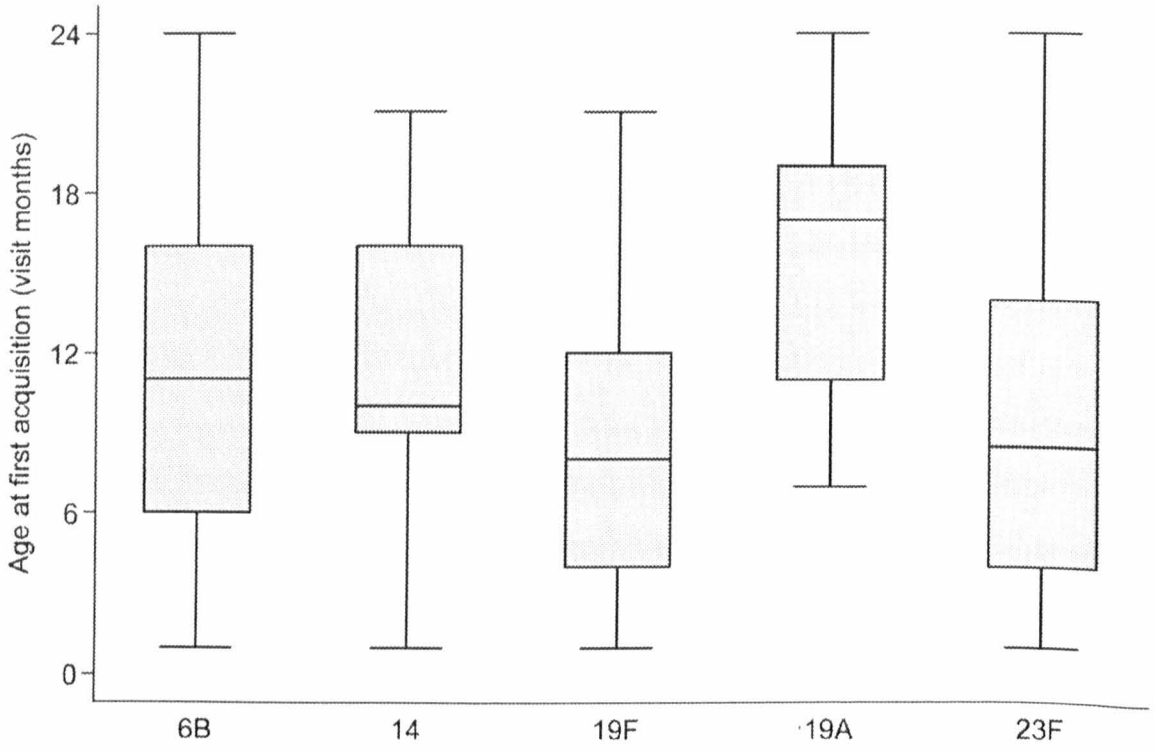


Figure 21. Age at first acquisition of five target pneumococcal serotypes

The graph summarises the monthly visit in which the serotype was detected in a nasopharyngeal swab for the first time in an infant.



5.1.5.2. Antibody kinetics

Serum IgG for all serotypes was highest in the first sample taken from the infants reflecting maternally derived, transplacentally transported IgG. Serum IgG antibody concentrations to all five serotypes declined between one and six months of age, with antibodies to serotype 14 having a significantly greater fold-change than the other serotypes ($P = .02$) (Figure 22, Table 27 & Table 28). Following this nadir, IgG concentrations increased modestly until 24 months, although none of the GMCs at 24 months were equal to the one month values (Figure 22 & Table 27). Between six and 24 months of age, the greatest increase was seen in IgG antibodies to 19A (mean fold-change 4.91 (95% CI 3.31 – 7.28) and the smallest in antibodies to serotype 14 (mean fold-change 0.91 (95% CI 0.54 – 1.52) ($P = .005$). At 24 months, measurable anti-capsular IgG

antibodies (≥0.15 μg/mL) were found in the sera of 19.4% to 97.1% of infants, varying considerably by serotype (Table 27).

Figure 22. Kinetics of serum IgG antibodies to five pneumococcal capsular polysaccharides in infants from 1 – 24 months of age

The shows geometric mean concentrations with 95% confidence intervals.

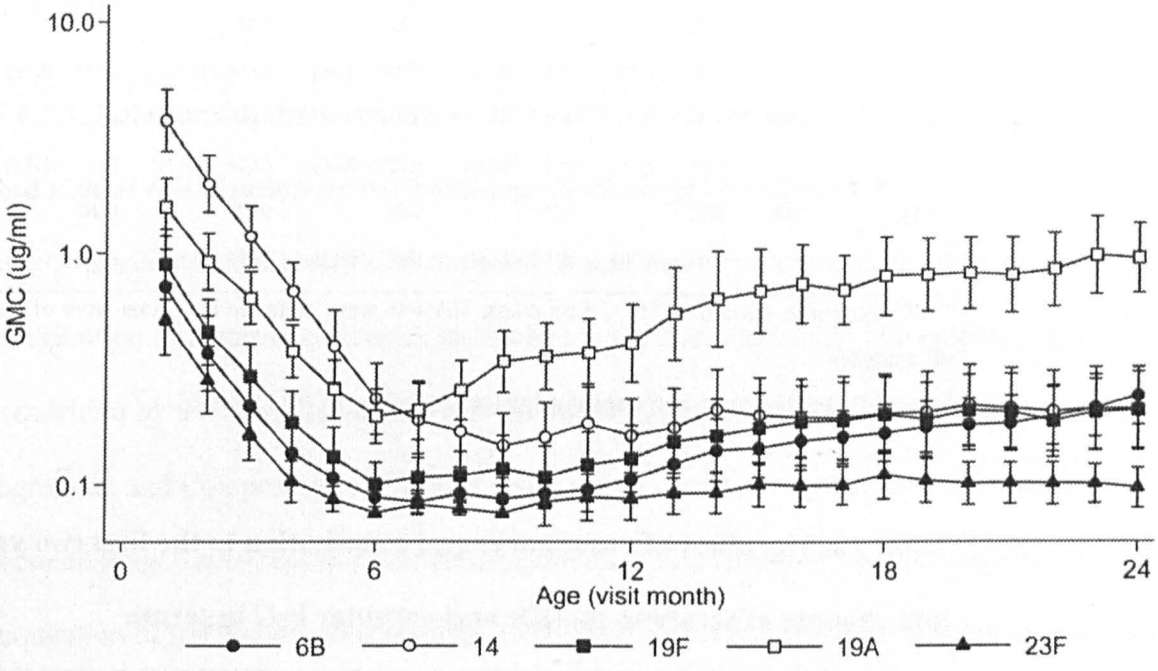


Table 27. Geometric mean serum anti-capsular IgG antibody concentrations in 36 infants during the first two years of life

Serotype	Sera (N)[a]	GMC (95% CI), µg/mL					Infants with detectable IgG at 24m, N (%)[b]
		1 month	6 months	12 months	18 months	24 months	
6B	858	0.71 (0.48 – 1.04)	0.09 (0.07 – 0.10)	0.11 (0.08 – 0.13)	0.17 (0.13 – 0.22)	0.24 (0.18 – 0.32)	28/36 (77.8)
14	858	3.74 (275 – 5.08)	0.23 (0.16 – 0.33)	0.16 (0.11 – 0.23)	0.20 (0.14 – 0.30)	0.21 (0.14 – 0.32)	18/36 (50.0)
19F	857	0.90 (0.63 – 1.27)	0.10 (0.08 – 0.12)	0.13 (0.09 – 0.18)	0.20 (0.14 – 0.29)	0.21 (0.14 – 0.31)	16/36 (55.6)
19A	857	1.58 (1.19 – 2.11)	0.20 (0.15 – 0.26)	0.41 (0.28 – 0.60)	0.80 (0.54 – 1.17)	0.95 (0.67 – 1.35)	34/35 (97.1)
23F	859	0.52 (0.36 – 0.74)	0.08 (0.08 – 0.08)	0.09 (0.07 – 0.11)	0.11 (0.09 – 0.14)	0.10 (0.08 – 0.12)	7/36 (19.4)

[a] All time points combined (1 – 24 months); 864 sera were included but some were of insufficient volume for all analyses

[b] Defined as a 24 month anti-capsular IgG concentration of ≥0.15 µg/mL

Table 28. The effect of nasopharyngeal colonisation in the first two years of life on fold changes of serotype-specific anti-capsular IgG in serum

Serotype	Exposure[a]	Six months			Twenty four months		
		Infants (N)	IgG fold-change[b] Mean (95% CI)	*P*[d]	Infants (N)	IgG fold-change[c] Mean (95% CI)	*P*[d]
6B	Colonised	5	0.13 (0.04 – 0.46)	.9	15	3.78 (2.07 – 6.90)	.09
	Non-colonised	31	0.12 (0.09 – 0.17)		21	2.18 (1.50 – 3.18)	
14	Colonised	2	0.03 (0.01 – 0.18)	.3	13	3.64 (1.66 – 7.97)	< .001
	Non-colonised	34	0.07 (0.05 – 0.10)		23	0.41 (0.27 – 0.64)	
19F	Colonised	8	0.11 (0.06 – 0.19)	.9	22	2.60 (1.56 – 4.33)	.2
	Non-colonised	28	0.12 (0.07 – 0.18)		14	1.50 (0.68 – 3.32)	
19A	Colonised	0	-	-	7	6.77 (2.93 – 15.63)	.4
	Non-colonised	36	0.12 (0.09 – 0.18)		29	4.53 (2.85 – 7.21)	
23F	Colonised	7	0.18 (0.07 – 0.46)	.5	18	1.46 (1.03 – 2.08)	.2
	Non-colonised	29	0.14 (0.09 – 0.21)		18	1.14 (0.93 – 1.38)	

[a] Colonised: detection of the pneumococcal serotype in the nasopharynx from 1 month to either 6 or 24 months

[b] 6 month serum IgG concentration divided by the 1 month serum IgG

[c] 24 month serum IgG concentration divided by the 6 month serum IgG concentration

[d] Comparison of logged fold-changes in serum IgG concentration in colonised vs. non-colonised infants

In the context of PCV administration, it has been suggested that a serum anti-capsular IgG concentration of ≥0.35 μg/mL protects against invasive disease caused by the homologous serotype (293, 294). By 24 months of age the proportion of infants with anti-capsular IgG concentrations above this threshold varied considerably by serotype: 5.6% (95% CI 0.1 – 18.7) for 23F, 30.6% (95% CI 16.3 – 48.1) for 19F, 33.3% (95% CI 18.6 – 60.0) for 6B, 38.9% (95% CI 23.1 – 56.5) for 14, and 85.7% (95% CI 67.2 – 93.6) for 19A ($P < .001$).

5.1.5.3. Relationship between maternally derived serum IgG anti-capsular antibodies and risk of colonisation by the homologous serotype in infancy

Specimens collected from the infant at one month of age were used to assess the contribution of maternally derived antibodies to the risk and timing of nasopharyngeal acquisition by the homologous serotype in the first two years of life. In univariate logistic regression and Cox proportion hazards models there were no significant associations between logged anti-capsular IgG concentrations at one month of age and subsequent acquisition of the homologous serotype (Table 29). Interestingly, the relationship between one month anti-19A antibodies and risk, and timing, of 19A acquisitions was the opposite of the other serotypes. A higher one month anti-19A IgG concentration was associated with higher risk, and earlier acquisition, of 19A although the numbers were small and statistical significance was not achieved.

Table 29. Assessment of the effect of maternally derived serum anti-capsular IgG antibodies on the risk and timing of nasopharyngeal acquisition of the homologous serotype in the infant over the first 24 months of life

Logged serum anti-capsular IgG concentrations in the infant specimen collected at one month of age were compared with homologous serotype acquisition in univariate logistic regression and Cox proportional hazards models. An OR of <1 indicated a lower risk of nasopharyngeal acquisition with increasing concentrations of anti-capsular IgG. A HR of <1 indicated a longer interval to acquisition with increasing concentrations of anti-capsular IgG.

Serotype	Risk of acquisition OR (95% CI)	*P*	Timing of acquisition HR (95% CI)	*P*
6B	1.06 (0.23 – 4.91)	.9	0.99 (0.32 – 3.11)	1.0
14	0.54 (0.07 – 3.85)	.5	0.65 (0.14 – 3.00)	.6
19F	0.64 (0.11 – 3.63)	.6	0.77 (0.32 – 1.81)	.5
19A	9.86 (0.46 – 213.16)	.1	6.49 (0.46 – 91.35)	.2
23F	1.91 (0.37 – 9.75)	.4	1.41 (0.46 – 4.34)	.5

5.1.5.4. Relationship between colonisation and serum IgG anti-capsular antibodies

Serum anti-capsular IgG concentration fold-changes (between one and six or six and 24 month specimens) were compared by grouping infants by colonisation status for the homologous serotype (Table 28). Colonisation by the sixth month visit was not associated with a significant increase in homologous serum anti-capsular IgG concentration over the same time period, compared with the non-colonised group. The mean anti-capsular IgG fold-change between six and 24 months was higher in the those infants who had been colonised at any time point compared with the non-colonised group for serotypes 6B, 19F, 19A, and 23F, although the differences were not statistically significant. However, for serotype 14 a significant increase was observed in the colonised group (3.64 vs. 0.41 μg/mL, $P < .001$). There were no significant differences between the proportions of infants with 24 month serum anti-capsular IgG concentrations ≥0.35 μg/mL by colonisation status,

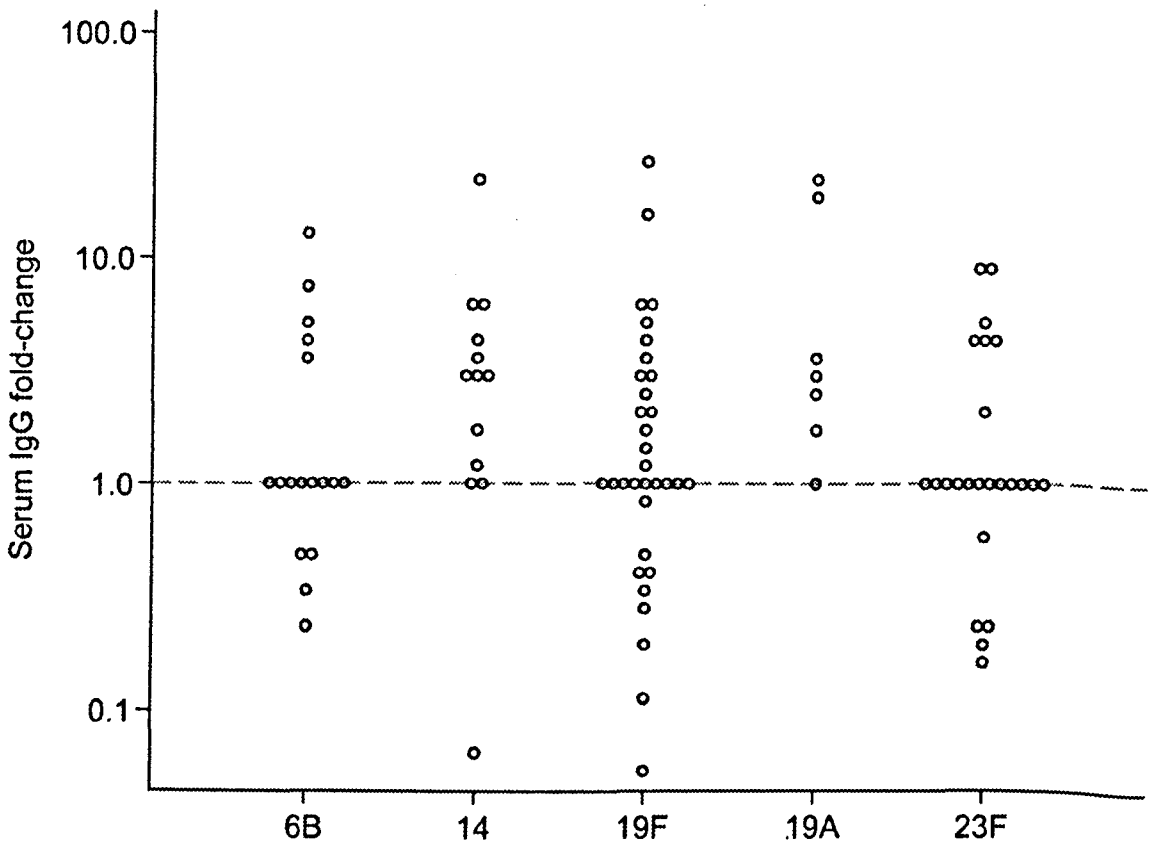
with the exception of serotype 14. For this serotype, 76.9% (10/13) of colonised infants had "protective" levels of serum IgG compared with 17.4% (4/23) of non-colonised infants ($P = .001$).

Within individuals, there was a significant correlation between 6 – 24 month fold-changes for anti-19F and anti-19A ($r = 0.47$, 95% CI 0.17 – 0.70). These were also correlated with the fold-change for anti-6B (6B & 19F: $r = 0.56$, 95% CI 0.28 – 0.75; 6B & 19A: $r = 0.54$, 95% CI 0.25 – 0.74).

5.1.5.5. Antibody responses to individual pneumococcal serotype acquisitions

The development of serotype specific antibodies following individual nasopharyngeal acquisitions was explored. The fold-change in serum anti-capsular IgG concentration was calculated between the visit prior to the sampling time point where nasopharyngeal acquisition was identified and the visit three months following the acquisition time point (i.e. the antibody fold-change over a four month time span). This fold-change could be calculated for 94/106 (88.7%) of the target serotype acquisitions: those acquisitions occurring up to and including the 21-month nasopharyngeal sampling point. Seven acquisitions of serotype 23F (7/24, 29.2%), 5/17 (29.4%) of 6B, 16/33 (48.5%) of 19F, 10/13 (76.9%) of 14, and 7/7 (100%) of 19A were associated with a measurable increase in homologous serum anti-capsular IgG titre (Figure 23).

Figure 23. Fold-changes in serum anti-capsular IgG concentrations in response to pneumococcal acquisition, by serotype



In the acquisitions where an increase in homologous anti-capsular IgG antibody titre was detected, the mean fold-changes were 6.00 (95% CI 3.21 – 11.23) for 6B, 3.86 (95% CI 2.17 – 6.88) for 14, 3.45 (95% CI 2.16 – 5.51) for 19F, 4.08 (95% CI 1.40 – 11.85) for 19A, 4.83 (95% CI 3.11 – 7.52) for 23F ($P = .7$). Combining data for all five serotypes in a linear regression model with robust standard errors to account for multiple acquisitions per infant, older age at acquisition was associated significantly with an increase in homologous anti-capsular IgG antibody titre ($\beta = 0.05$; 95% CI 0.03 – 0.07; $P < .001$) controlling for serotype reacquisitions within an individual (Figure 24). Considering serotypes individually, this association remained significant for 6B, 19F and 23F ($P = .03$, .02, and < .001, respectively) (Figure 24). Due to the small sample size, the effect of reacquisition of a serotype on magnitude of anti-capsular IgG response could only be assessed for 19F (22 primary acquisitions and 15 reacquisitions). Although the mean fold-

change was greater for reacquisitions than primary acquisitions (2.07 vs. 0.97; P =.1), this effect was not significant when controlling for age in the linear regression model (Figure 25).

Figure 24. Fold-changes in serum anti-capsular IgG concentrations in response to pneumococcal acquisition, by age and serotype

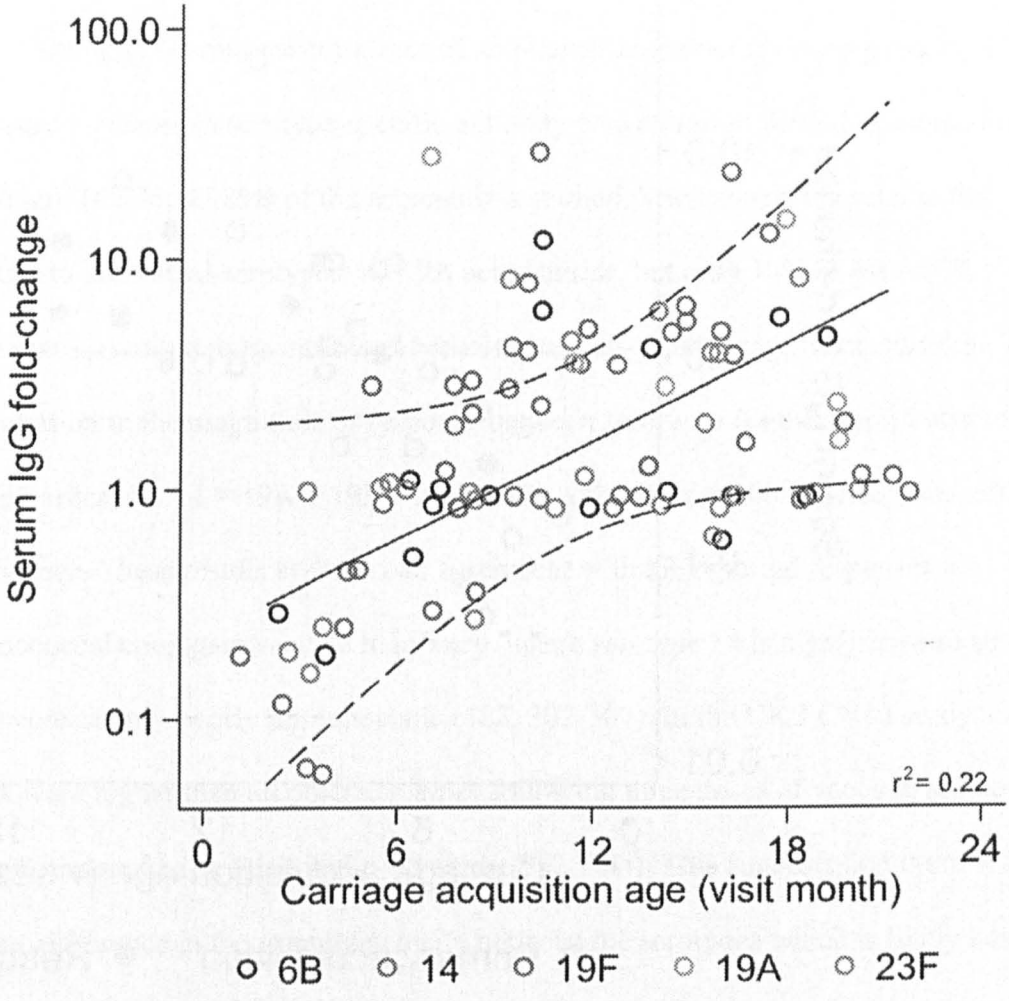
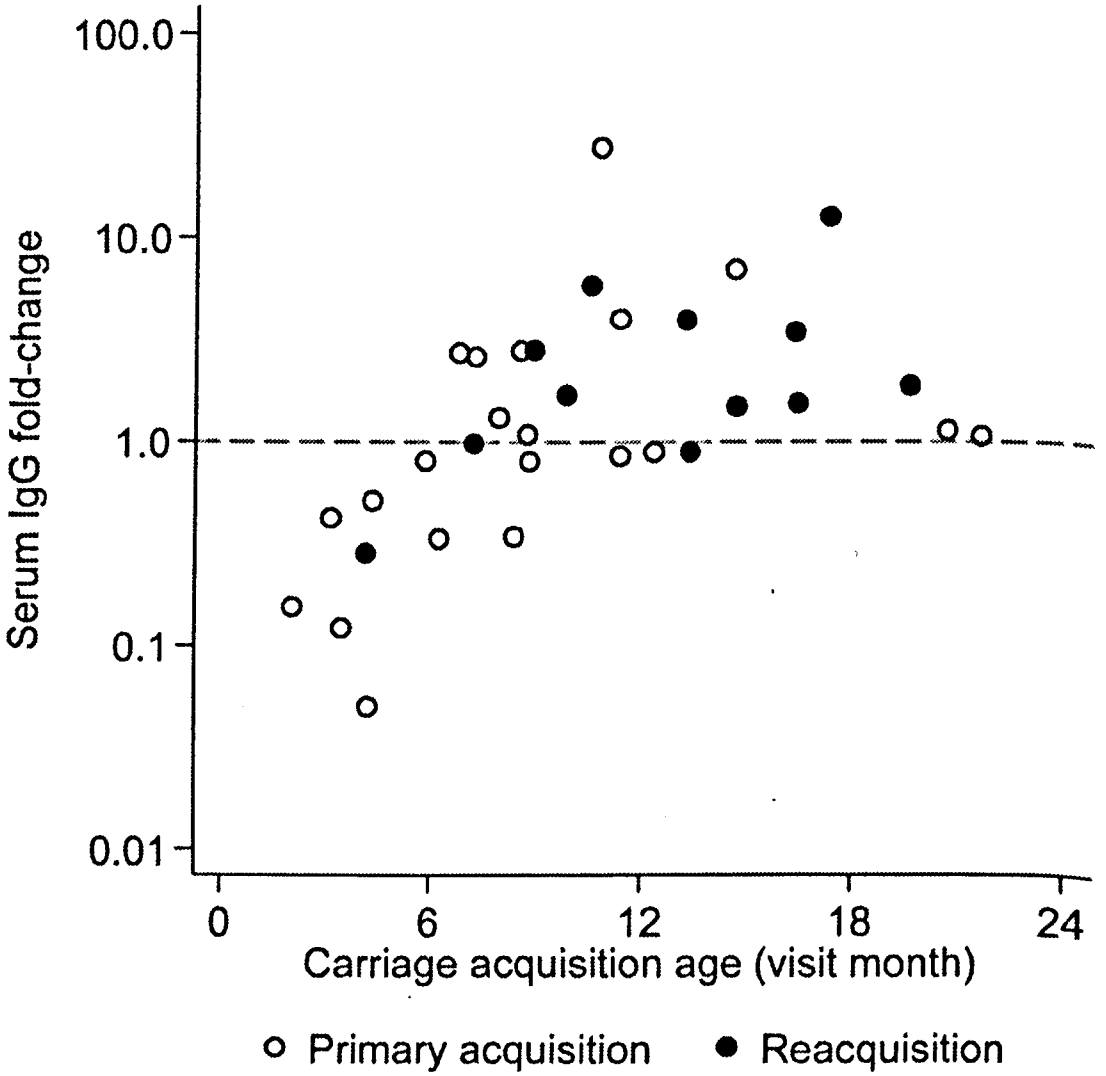


Figure 25. Fold-changes in serum anti-capsular IgG concentration in response to nasopharyngeal acquisitions of 19F

Primary acquisitions (i.e. an infant's first acquisition of 19F) are shown as hollow circles and reacquisitions of 19F, following at least two consecutive months without documented carriage, are shown as filled circles.



Antibody response over alternative time spans of one, two, or three months were assessed as part of this analysis: there were no significant differences in IgG fold-changes within each serotype when these were compared. However, correlation coefficients for fold-change versus acquisition age were superior using the four month interval (data not shown).

5.1.6. Discussion

The study objective was to examine the serum anti-capsular IgG responses to nasopharyngeal acquisition of common pneumococcal serotypes in a population of children aged less than two years, in whom pneumococci were carried frequently. Intensive sampling and the use of the latest generation of anti-capsular polysaccharide IgG detection ELISA permitted refinement of previous findings regarding the immunogenicity of nasopharyngeal pneumococcal colonisation in this age group (188, 190, 301).

Although serum concentrations of anti-capsular antibodies were generally low, detectable increases in serotype-specific antibody concentration were documented in almost half (45/94; 47.8%) of the acquisitions studied. Variations were seen in the response to individual serotypes: all 19A acquisitions, but only 30% of 6B or 23F acquisitions, resulted in an increased homologous anti-capsular IgG concentration. There was variation in the magnitude of response between serotypes (in the second year of life the rank order was 14 > 19A > 19F > 23F > 6B), although this did not reach statistical significance. These results are in broad agreement with the reported responses to pneumococcal conjugate vaccine in infancy, where serotype 14 is highly immunogenic and 23F is consistently poorly immunogenic (182, 302-307). In the UK PCV13 study, GMCs to 19A were higher than all other serotypes following three doses of vaccine, although this serotype was ranked seventh in the US study (302, 304). This suggests that there is an intrinsic difference in the immunogenicity between the serotypes which is likely based on chemical structure.

As expected, there was a positive association between antibody response and age at pneumococcal acquisition, reflecting the maturation of the infant immune response. Infants colonised by serotype 14 had a significantly greater increase in specific anti-capsular IgG between six and 24 months of age than non-colonised infants, and a similar trend was observed for other serotypes studied. The number of infants studied is likely to have limited the power to detect small differences between colonised and non-colonised

individuals for the less immunogenic capsular types. It was possible to examine the anti-capsular IgG responses to both primary nasopharyngeal acquisitions and reacquisitions of serotype 19F. Increasing age, rather than previous exposure, defined the magnitude of response for this serotype. Although, the numbers were small, there was no evidence of immune hyporesponsiveness as a result of previous exposure to 19F when reacquisition of this serotype occurred. Given the variation in immunogenicity between capsules, this finding may not be generalisable to all serotypes and is in contrast to the finding that nasopharyngeal colonisation impacts on the magnitude of subsequent serotype specific vaccine responses (308, 309).

The study results agree in part with findings from previous infant cohort studies. Gray and co-workers observed no differences between serum anti-capsular IgG concentrations, for serotypes 3, 6, 14, and 23, in carriers versus non-carriers at 12 and 24 months of age. They did, however, observe serum anti-capsular IgG responses to colonisation and infection, particularly when these occurrences represented re-exposure to a given serotype, although it is important to note that the methodology used to determine antibody concentrations in these studies has been superseded by more accurate assays (188, 301). In the Finnish birth cohort study, infants with previous exposure to serotype 11A or 14 pneumococci had a significantly higher homologous anti-capsular GMC at 24 months of age compared with non-exposed infants (190). A correlation between exposure and homologous anti-capsular IgG concentration at 24 months of age was not observed for the other serotypes studied (6B, 19F, and 23F). Infants were found to have anti-capsular antibody responses to non-carried pneumococcal serotypes, a finding possibly related to the ELISA methodology, in particular the absence of 22F pre-adsorption of the sera prior to analysis.

At 24 months, a proportion of infants had serum anti-capsular IgG concentrations above the putative threshold for protection against invasive disease (0.35 μg/mL) and this ranged from only 5.6% of infants for 23F to 85.7% of infants for 19A. Serotype 14 was the

only serotype in which a significantly greater proportion of colonised infants had protective anti-capsular IgG concentrations compared to non-colonised infants at 24 months of age. Notwithstanding the limitations around the derivation of the correlate of protection quoted here, these data support the hypothesis that, although anti-capsular antibodies are an important defence against invasive pneumococcal infection, other immune mechanisms, perhaps mucosal antibody produced by mucosal B cells or T cells, are also critical for the observed decline in incidence for invasive disease caused by all common serotypes after the first year of life (310). There was no evidence to suggest that maternally derived anti-capsular antibodies reduced the risk, or delayed the timing, of acquisition of any of the serotypes studied. Previous work has suggested that a serum anti-capsular IgG concentration of at least 5 μg/mL might be required to protect against colonisation in adults, a level higher than the GMCs at one month of age for all serotypes studied in the current cohort (184). However, few of the infants studied acquired any of the target serotypes before six months of age, limiting the possibility of detection of an effect of maternal antibodies on nasopharyngeal acquisition.

Within individuals, significant correlation was found for fold-changes of serum anti-19F and 19A IgG, and also for anti-6B and anti-19F/A IgG concentrations, between six and 24 months of age. The correlation between anti-6B and anti-19F was not unexpected, given that these serotypes were carried at high frequency in the study infants with 19 (52.8%) infants being colonised by either 6B or 19F and nine (25%) infants being colonised by both serotypes. With the exception of 19A, 6 – 24 month specific anti-capsular IgG fold-changes were minimal in non-colonised individuals implying that little cross-reactive antibody was produced. Serotype 19A was carried relatively infrequently (seven (19.4%) infants) but 19A specific IgG was detected in most infants, most probably as a result of antibodies induced by 19F colonisation cross-reacting with 19A as seen after vaccination with PCV7 (311).

A limitation of the study was that determination of serum IgG levels alone does not provide a complete picture of the humoral immune response to pneumococcal colonisation. Measurement of mucosal IgA antibody concentration as well as determination of antibody function, including assessments of avidity and opsonophagocytic activity (OPA), would have been desirable (312, 313). However, to date, correlates of protection against invasive disease and colonisation have only been established for serum IgG concentrations (184, 293).

In conclusion, this study has demonstrated that nasopharyngeal acquisition of pneumococci frequently results in a measurable serum IgG response in infants from six months of age. By 24 months of age almost 90% of infants had anti-capsular IgG concentrations above the putative protective level for certain serotypes. However, anti-capsular responses varied considerably by serotype although generally increased in magnitude with age. Further work on the immunological mechanisms underlying the protection from nasopharyngeal acquisition is required.

5.2. Serum antibody responses to a large panel of pneumococcal surface protein antigens in the first two years of life

5.2.1. Summary

Background

Next generation vaccines against *S. pneumoniae* are likely to include pneumococcal surface or virulence protein antigens, since these could potentially protect against all pneumococcal serotypes. Assessment of antibody responses to nasopharyngeal colonisation in early childhood may aid vaccine antigen selection.

Methods

Nasopharyngeal swabs and serum specimens were collected from birth until 24 months of age in 234 mother-infant pairs. Swabs were cultured to detect pneumococcal colonisation. Serum IgG titres to 27 pneumococcal protein antigens were measured in 2,264 sera.

Results

Antibodies to all protein antigens were detectable in every maternal serum specimen. Transplacental antibody transfer was efficient with mean cord:mother titre ratios between 0.77 - 1.08. Titres to four proteins (LytB, PcpA, PhtD, and PhtE) were significantly higher in mothers colonised by pneumococci at delivery. Maternally-derived antibodies to PiuA and Spr0096 were associated with delayed pneumococcal acquisition in infants in univariate Cox proportional hazards models; however this effect was lost in a multivariate model including environmental and household factors. In infants, antibodies to several proteins (LytC, PhtD, PhtE, PspA, and SP2027) had weak positive correlations with total duration of pneumococcal colonisation or number of new acquisitions. No evidence of serum IgG-mediated protection from nasopharyngeal colonisation was detected.

Conclusions

Nasopharyngeal colonisation in young children resulted in demonstrable serum IgG responses to pneumococcal surface and virulence proteins. However, antibodies to these

proteins did not protect against colonisation in an environment where there was frequent exposure to pneumococci.

5.2.2. Introduction

Currently available pneumococcal conjugate vaccines have been shown to significantly reduce the incidence of invasive disease, pneumonia, and otitis media caused by most of the serotypes covered included in the vaccine, as well as reduce all-cause mortality (3-6, 314). The relative increase in incidence of invasive disease by serotypes not included in the vaccines reflects changes in nasopharyngeal pneumococcal colonisation following vaccine introduction, since it has been demonstrated that PCVs reduce colonisation by vaccine serotypes and that non-vaccine serotypes might then appear in the nasopharynx (67, 68). The next generation of pneumococcal vaccines would ideally protect against all serotypes by targeting conserved cell surface or virulence proteins. Vaccines containing pneumococcal proteins have been demonstrated to provide protection against invasive pneumococcal disease in mouse models. Proteins that have been evaluated as vaccine antigens include the pneumococcal pilus (PI-1, comprising three subunits RrgA, RrgB, and RrgC), pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumococcal surface protein C / choline binding protein A (PspC/CbpA) pneumolysin (Ply), histidine triad proteins (PhtA-E), neuraminidase (NanA), iron transport proteins (PiuA, PiaA) and also novel proteins, such as a protein required for cell wall separation of group B streptococcus (PcsB) and serine/threonine protein kinase (StkP), discovered by ANTIGENome scanning (194, 195).

Nasopharyngeal colonisation has been demonstrated to result in demonstrable serotype-specific antibody responses in adults (184), although antibody responses to the polysaccharide capsule in young children are more modest (84, 190). Colonisation by a vaccine serotype at the time of PCV immunisation has been shown to result in reduced vaccine-induced serum anti-capsular IgG concentrations to the homologous serotype (308).

As part of the pneumococcal protein vaccine development process, assessments of the relationship between nasopharyngeal colonisation and antigen-specific immune responses in young children are critical and may predict whether carriage-mediated vaccine hyporesponsiveness might be an issue. Previous studies have shown that antibodies to several pneumococcal protein vaccine candidates are measurable in adult human sera and that pneumococcal contact in early childhood, either colonisation or otitis media, generally results in serum IgG antibody responses, although these are variable by protein and population (71, 111, 198-201, 203-211, 315-317). A study of experimentally induced pneumococcal colonisation in adults demonstrated significant serum IgG responses to both CbpA and PspA (213) while antibodies to the N-terminal of PspA were shown in the same experimental set up to protect against colonisation (212). To date however, most studies have been cross-sectional, have described responses to a small number of protein antigens, or included limited sampling points, particularly in the second year of life where pneumococcal colonisation remains common and the infant immune system continues to mature.

5.2.3. Aims and objectives

The aim of this study was to examine the temporal relationships between pneumococcal nasopharyngeal colonisation and development of serum IgG to pneumococcal surface and virulence proteins over the first two years of life in a population where there is early and sustained pneumococcal carriage in infancy. The effect of transplacentally transferred IgG antibodies to these proteins on the timing of first pneumococcal colonisation in infants was also explored.

5.2.4. Methods

5.2.4.1. Study site and population

Specimens collected from mothers and infants from the "immunology" follow-up group of the Maela cohort were selected for the current study. At delivery, a nasopharyngeal swab (NPS) and serum specimen was collected from the mother and a serum specimen obtained from the umbilical cord. After delivery, NPS were taken from both mother and infant at monthly intervals, from 1 – 24 months of age. At these visits, a serum sample was also collected from the infants. No further serum specimens were obtained from the mother (section 2.2.3).

5.2.4.2. Detection of pneumococcal nasopharyngeal colonisation

Nasopharyngeal swabs were collected and processed according the standard WHO protocol, as described in sections 2.2.4.1 and 2.2.5.1 (70, 291). All pneumococcal isolates were serotyped by latex agglutination with Quellung confirmation of equivocal results (273).

5.2.4.3. Antigens and serologic methods

Antibodies were measured using a direct binding electrochemiluminescence-based multiplex assay adapted for the simultaneous quantitation of IgG to 27 selected pneumococcal antigens (Table 30). These proteins were generously donated by GlaxoSmithKline (GSK), Intercell AG, Novartis, PATH, Sanofi Pasteur, and Prof Susan Hollingshead (University of Alabama).

The assay was based on that described for pneumococcal polysaccharide antigens utilising MesoScale Discovery (MSD) technology which employs disposable multispot microtitre plates (multiarray plates; MSD, Gaithersburg, MD) that include integrated screen-printed carbon ink electrodes on the bottom of the wells (318). Antigens were bound directly to the carbon surface and IgG from serum was detected using a

chemiluminescent antibody. The pneumococcal reference serum 007 was used as a standard on each plate and assigned a value of 1000 arbitrary units for each antigen (319). Antibody levels in unknown sera were expressed as a titre with reference to the amount in 007.

Table 30. Protein antigens assessed

Name	Int. ID	Protein details	Donor
CbpA	PP01	Choline binding protein A, without choline binding domain (CbpA)	GSK
LytB	PP11	Endo-beta-N-acetylglucosaminidase (LytB)	Sanofi Pasteur
LytC	PP02	Lysozyme (LytC C-ter)	GSK
NanA	PP33	Neuraminidase (NanA)	UAB
PcpA	PP13	Choline binding protein (PcpA)	Sanofi Pasteur
PcsB-1	PP06	Secreted 45 kDa protein (PcsB, SP2216-1)	Intercell
PcsB-2	PP32	Secreted 45 kDa protein (PcsB, Spr2021)	Novartis
PhtD-1	PP03	Pneumococcal histidine triad D (PhtD)	GSK
PhtD-2	PP14	Pneumococcal histidine triad protein (PhtD)	Sanofi Pasteur
PhtE	PP10	Truncated histidine triad protein (PhtE-T1)	Sanofi Pasteur
PiaA	PP09	Part of iron uptake ABC transporter (PiaA)	PATH
PiuA	PP08	Part of iron uptake ABC transporter (PiuA)	PATH
Ply-1	PP12	Pneumolysin (Wt-Ply)	Sanofi Pasteur
Ply-2	PP17	Pneumolysin (Ply)	UAB
PsaA	PP04	Pneumococcal surface adhesin A (PsaA, SP1650)	Intercell
PspA-Fam1	PP16	Pneumococcal surface protein A, family 1 (PspA)	UAB
PspA-Fam2	PP15	Pneumococcal surface protein A, family 2 (PspA)	UAB
RrgA-T4	PP22	RrgA pilus subunit, adhesin (RrgA, TIGR4)	Novartis
RrgB-T4	PP18	RrgB pilus subunit, backbone (RrgB, TIGR4)	Novartis
RrgB-6B	PP19	RrgB pilus subunit, backbone (RrgB, serotype 6B)	Novartis
RrgB-23F	PP20	RrgB pilus subunit, backbone (RrgB, serotype 23F)	Novartis
StkP	PP05	Serine threonine kinase protein (StkP, SP1732-3)	Intercell
StrH	PP29	Beta-N-acetylhexosaminidase, (StrH, Spr0057)	Novartis
SP0609	PP31	Amino acid ABC transporter, amino acid-binding protein	Intercell
SP2027	PP07	Conserved hypothetical protein (Spr1 / SP2027)	Intercell
SP2194	PP30	ATP-dependent Clp protease, ATP-binding subunit	Intercell
Spr0096	PP24	LysM domain-containing protein	Novartis

5.2.4.4. Serum specimen selection

Specimens were selected to obtain good coverage at each sampling point during the first year of life and to include the time-points from the second year of life with the largest number of specimens. The following specimens were included:

- All mother delivery and cord specimens;

- All specimens from infants with a complete first year set of serum samples. In infants with a complete first year serum set, sera collected at the 18 and 24 month visits were also included;
- Specimens from the 6, 12, 18, and 24 month visits in the remaining infants, where all of these samples were collected;
- All six infant sera collected from the only infant to reach 24m without becoming colonised by *S. pneumoniae.*

5.2.4.5. Statistical analysis

Antibody titres were normalised by log-transformation prior to analyses. Student's t-test was used to compare antibody titres and *S. pneumoniae* colonisation status in mothers at delivery. The impact of cord blood antibody titres on the timing of first pneumococcal acquisition in infants was analysed by survival analysis. To analyse infant antibody kinetics, geometric mean titres and their 95% confidence intervals were calculated for each specimen time point, stratifying by infant colonisation status. An infant was included in the "colonised" group at every time point following the first isolation of *S. pneumoniae* from a NPS specimen. To summarise kinetics, GMT ratios were calculated by dividing the 6, 12, or 24 month GMT by the cord blood GMT. To explore the relationship between colonisation in infancy and serum IgG responses, Pearson correlation coefficients were determined for antibody titre fold-changes between 6 or 12 months and 24 months and pneumococcal colonisation duration (the number of swabs in which *S. pneumoniae* was isolated) or acquisitions (the first appearance, or re-appearance following ≥2 consecutive negative NPS specimens, of a pneumococcal serotype in the nasopharynx) in the first two years of life. Correlation coefficients were also determined for twelve month antibody titres and the number of acquisitions in the second year of life, in order to assess whether higher antibody titres at twelve months protected against colonisation in the second year of life.

5.2.5. Results

2,264 serum specimens from 230 mothers and 222 infants were included in the analyses. This selection comprised 230 mother delivery, 184 cord, and 1,850 infant serum specimens (Table 31).

Table 31. Serum specimens analysed

Study group	Time point (Infant age [m])	Number of sera
Mother	Delivery	230
Cord	Delivery	184
Infant	1	134
	2	134
	3	134
	4	133
	5	133
	6	150
	7	133
	8	132
	9	134
	10	132
	11	134
	12	149
	18	99
	24	119
Total		**2,264**

5.2.5.1. Maternal antibody titres and transplacental transfer

Two hundred and twenty nine mothers had paired serum and NPS specimens collected within seven days of birth: 20.1% (46/229) were colonised by *S. pneumoniae.* Every mother had measurable serum IgG antibodies to all proteins studied. Geometric mean antibody titres to four proteins were significantly higher in women who were colonised by *S. pneumoniae* at the time of delivery: LytB (1093.5 vs. 747.9, $P = .0002$); PcpA (1264.4 vs. 981.3, $P = .04$); PhtD, both PhtD-1 (830.3 vs. 613.7, $P = .02$) and PhtD-2

(758.1 vs. 576.4, $P = .02$); and PhtE (789.0 vs. 571.3, $P = .02$). There was a trend in the same direction for antibodies to Ply-1 (712.7 vs. 566.5, $P = .07$) and PsaA (1674.7 vs. 1216.9, $P = .05$).

There were 183 mother-cord blood specimen pairs. Transplacental antibody transfer was efficient: the mean ratio of cord to mother serum antibody titres was between 0.77 and 1.08 for all proteins.

5.2.5.2. Cord titres and time to first pneumococcal acquisition

There were 184 cord blood specimens analysed and the effect of cord serum antibody titres on the timing of first pneumococcal acquisition could be examined in 179 infants, since five infants were lost to follow-up after collection of the cord blood specimen. In these infants, the median time at first pneumococcal acquisition was 46 days (IQR 16 - 77) and all but one infant had been colonised by the 11 month visit.

In univariate Cox proportional hazards models, higher serum IgG antibody titres to two proteins, PiuA and Spr0096, were significantly associated with delayed pneumococcal acquisition (PiuA: HR 0.68, 95% CI 0.46 - 0.98), $P = .04$; Spr0096: HR 0.73, 95% CI 0.57 - 0.95, $P = .02$). There were similar trends (HR <1 but $P = .05 - .1$) for PcsB (both PcsB-1 & -2) and SP2027. There was no evidence of higher cord blood antibody titres being significantly associated with earlier pneumococcal acquisition for any of the proteins (i.e. HR >1).

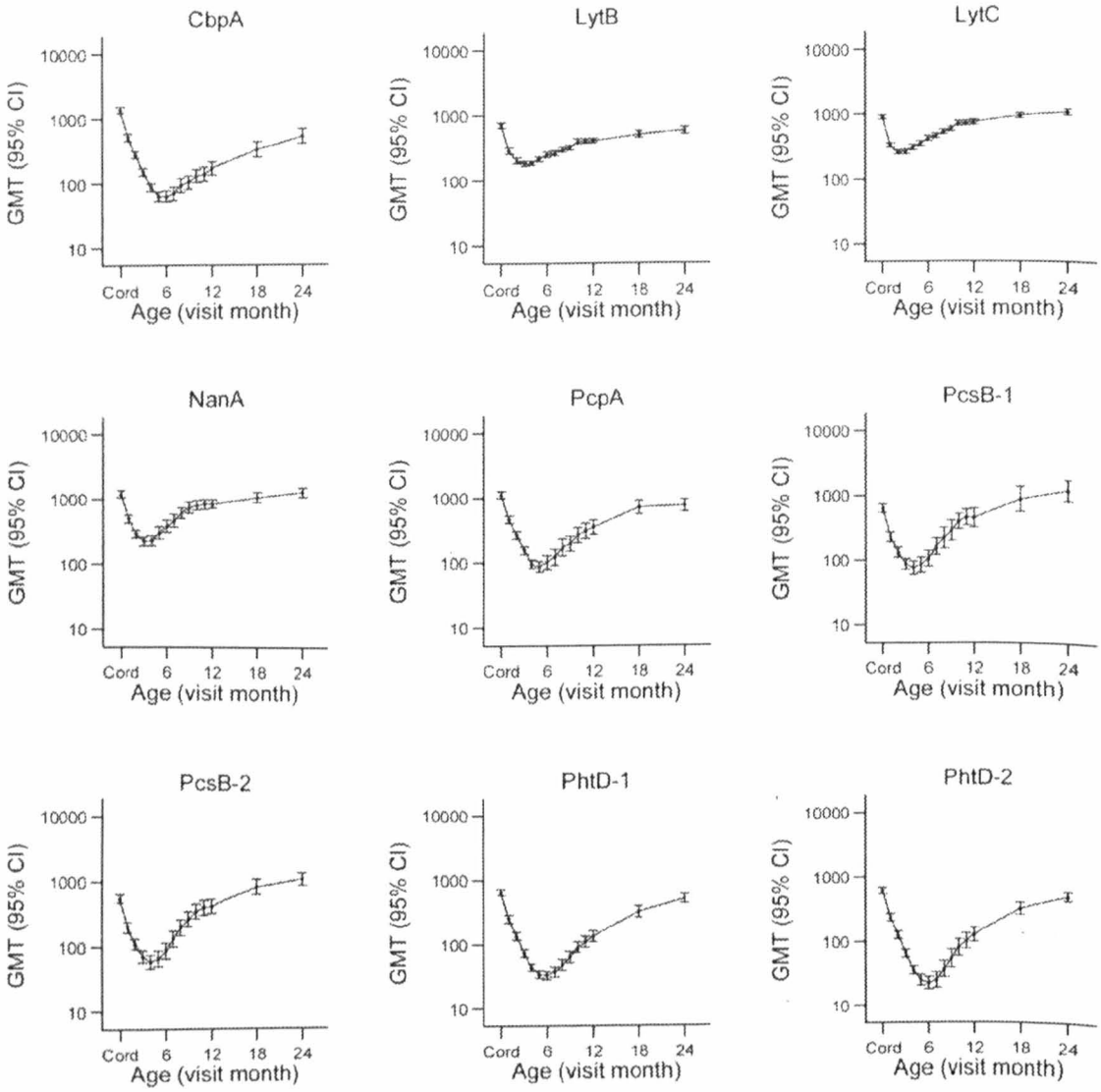
In a multivariate Cox model, which included environmental factors potentially associated with earlier infant pneumococcal acquisition, cord blood antibody titres to PiuA (HR 0.70, 95% CI 0.45 – 1.08, $P = .1$) or Spr0096 (HR 0.82, 95% CI 0.62 – 1.09, $P = .2$) were no longer significantly associated with timing of pneumococcal acquisition but maternal smoking (HR 1.49, 95% CI 1.01 – 2.21, $P = .046$) was significantly associated with earlier acquisition age with similar trends for maternal pneumococcal colonisation

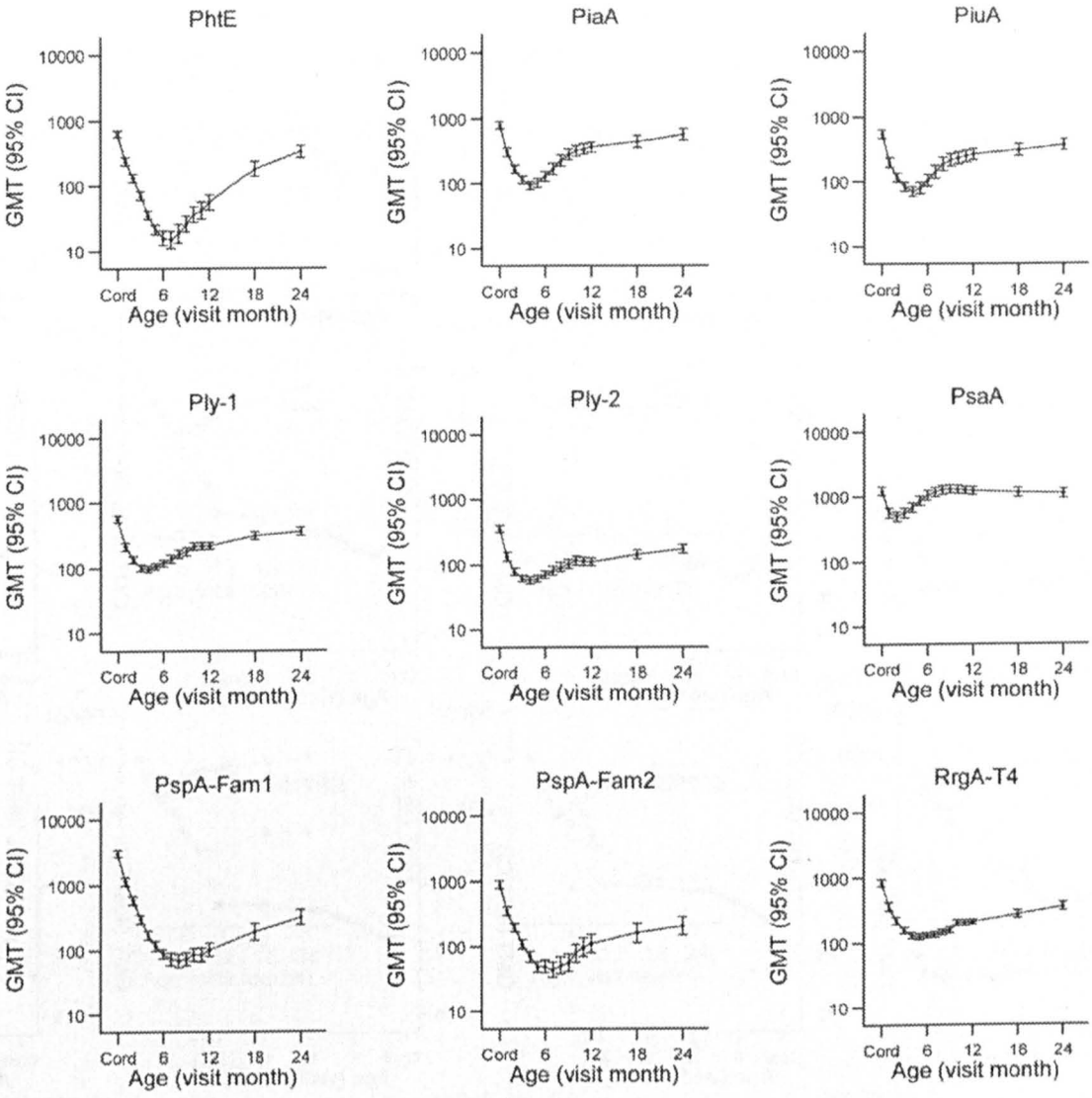
(HR 1.49, 95% CI 1.00 – 2.25, $P = .05$) and young children in the household (HR 1.39, 95% CI 0.99 – 1.96, $P = .06$).

5.2.5.3. Antibody kinetics in infants

In the 222 infants studied, 40.3% were colonised by pneumococci at the one month visit and between 66.4% and 88.0% were colonised at each subsequent time point. Geometric mean serum IgG antibody titres to all proteins fell rapidly after birth to a nadir at a median of 4 months (IQR 3 – 5; range: 2 – 8) (Figure 26). Subsequently, GMTs increased and this was largely in response to pneumococcal exposure, although kinetics varied considerably by protein (Figure 27). The ratio of GMT at birth (cord blood) to twelve months of age ranged from 0.03 to 1.08. The twelve-month GMT for four proteins had reached cord blood levels (ratio ≥1.0): PsaA, SP0609, SP2027, and SP2194. Serum IgG GMT ratios for the cord and 24 month specimens were between 0.10 and 2.00. The GMT ratio was >1.0 for seven proteins (LytC, NanA, PcsB (PcsB-1 & -2), SP0609, SP2027, SP2194, and Spr0096) and <0.5 for another seven (CbpA, PhtE, Ply-2, PspA (PspA-Fam1 & -Fam2), RrgA-T4, RrgB-23F, and StkP) (Figure 28). Evidence of increases in specific IgG in the absence of detectable colonisation was seen for certain proteins, most clearly pneumolysin (Ply-1 & -2) (Figure 27).

Figure 26. Geometric mean serum IgG antibody titres to pneumococcal proteins by age, with 95% confidence intervals




PhtE
PiaA
PiuA
Ply-1
Ply-2
PsaA
PspA-Fam1
PspA-Fam2
RrgA-T4
GMT (95% CI)
10000
1000
100
10
Cord
6
12
18
24
Age (visit month)

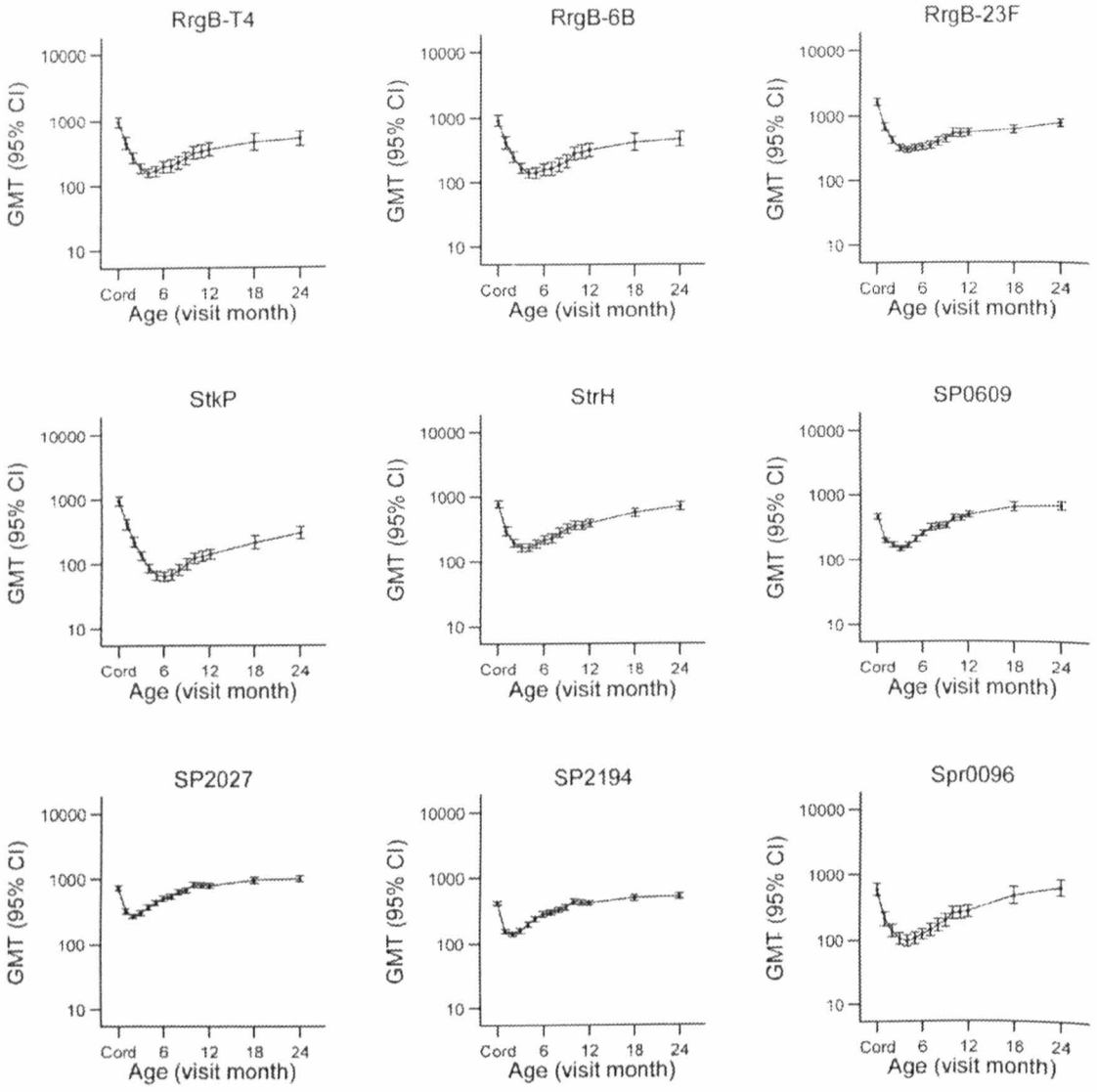
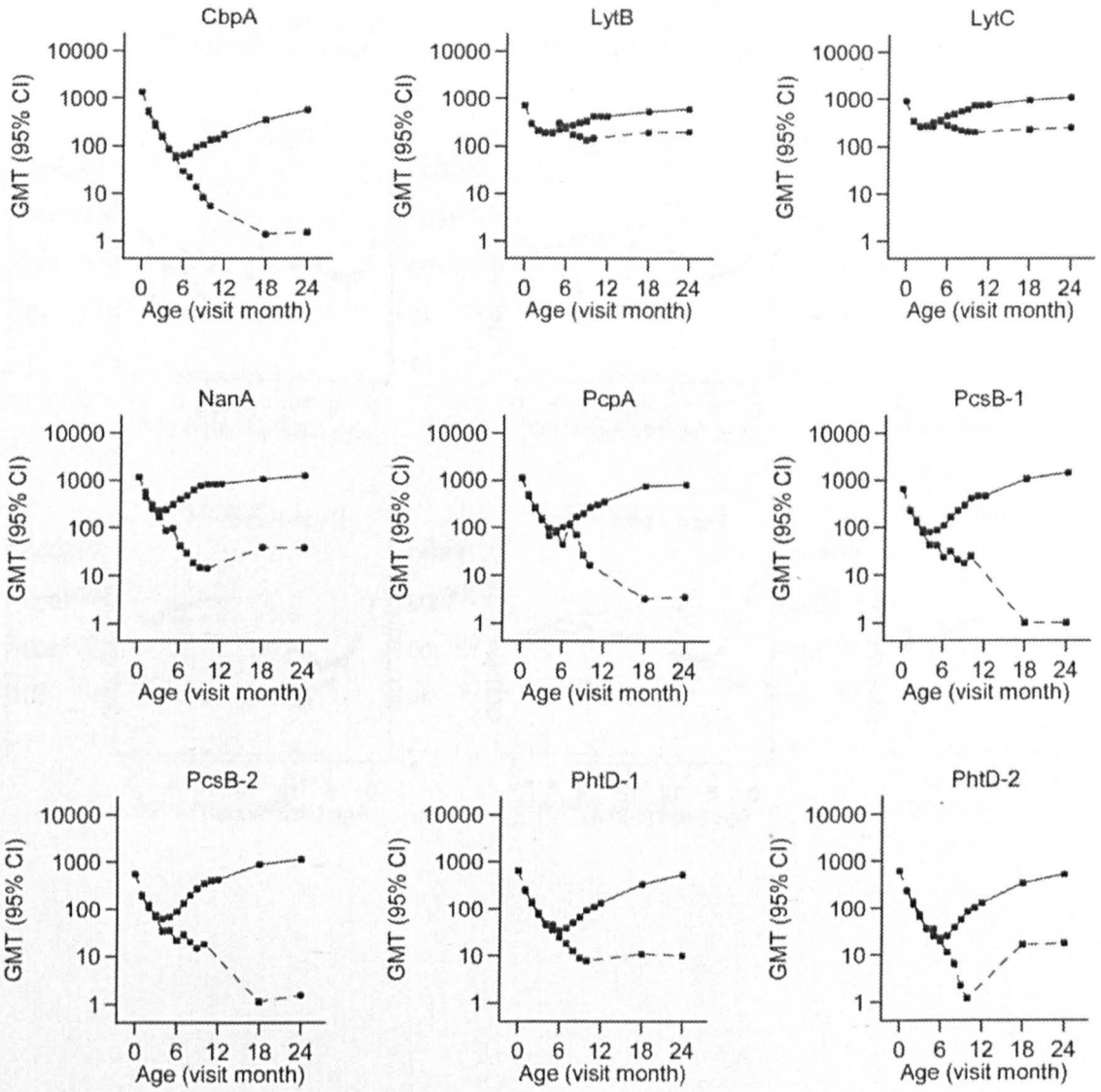
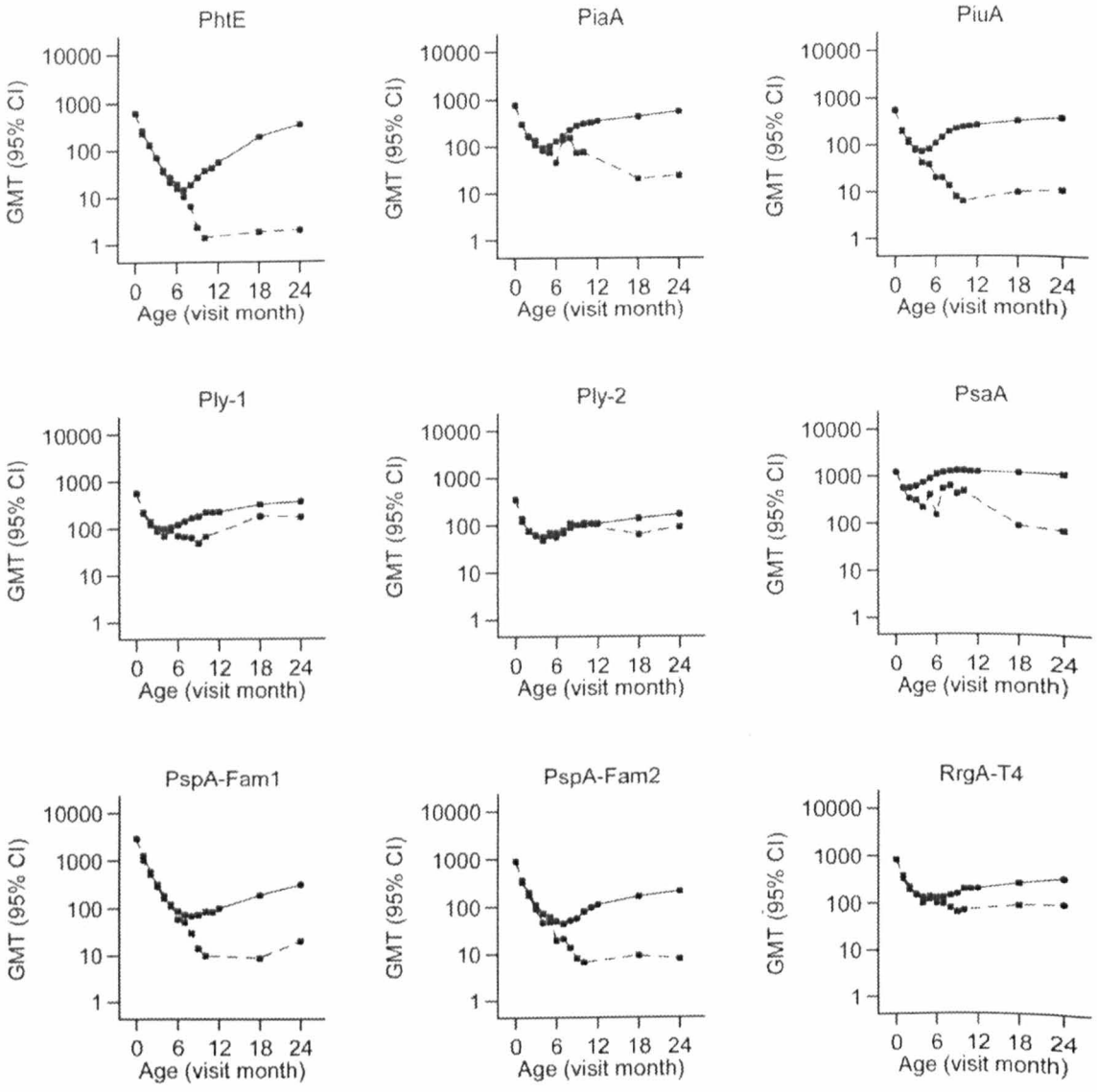

RrgB-T4
RrgB-6B
RrgB-23F
StkP
StrH
SP0609
SP2027
SP2194
Spr0096
GMT (95% CI)
10000
1000
100
10
Cord
6
12
18
24
Age (visit month)

Figure 27. Geometric mean serum IgG antibody titres to pneumococcal proteins, by age and pneumococcal colonisation status

Solid lines represent infants who had become colonised by *S. pneumoniae* and dashed lines represent infants who remained uncolonised. Age "0" indicates cord blood specimens.




PhtE
PiaA
PiuA
Ply-1
Ply-2
PsaA
PspA-Fam1
PspA-Fam2
RrgA-T4
GMT (95% CI)
10000
1000
100
10
1
0 6 12 18 24
Age (visit month)

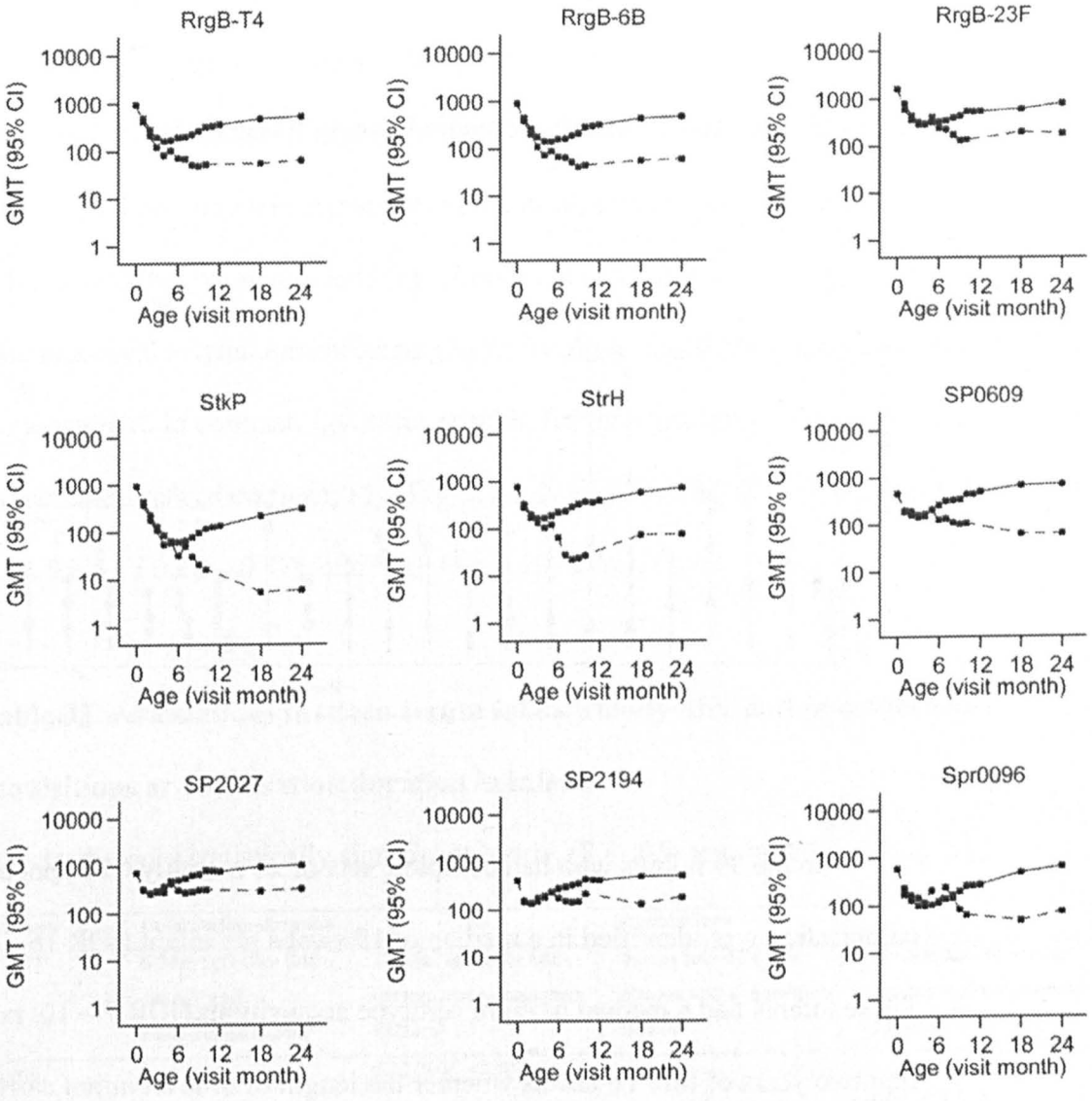
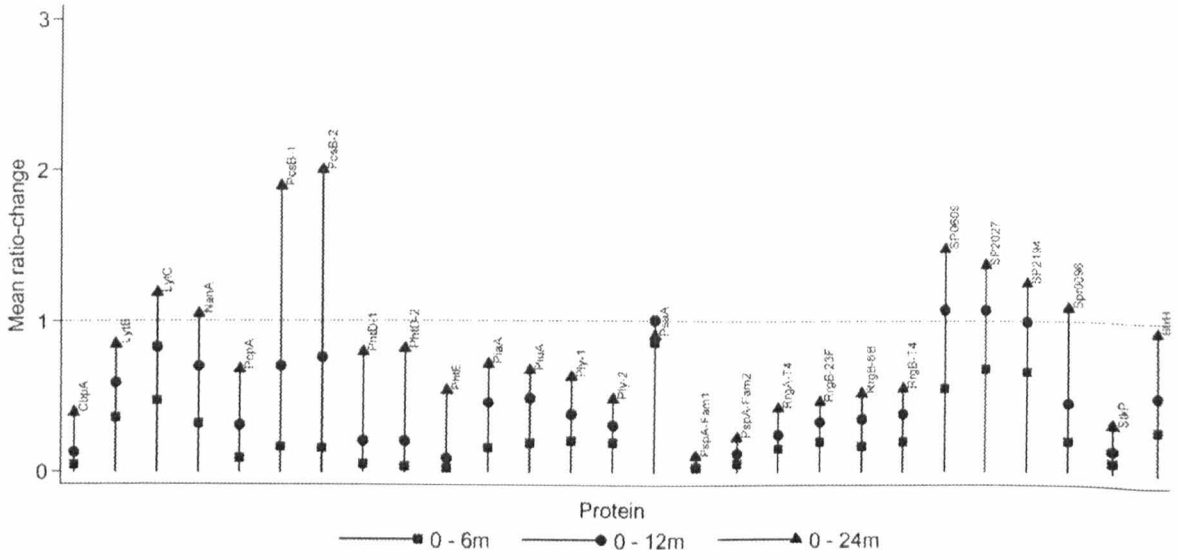

RrgB-T4
RrgB-6B
RrgB-23F
StkP
StrH
SP0609
SP2027
SP2194
Spr0096
GMT (95% CI)
10000
1000
100
10
1
0 6 12 18 24
Age (visit month)

Figure 28. Mean fold-changes in serum IgG antibody titres in the first two years of life

Titres at 6, 12, and 24 months compared to cord blood titres.



In the 99 infants who had complete sets of 24 monthly NPS, pneumococcal colonisation was identified in a median of 19 swabs per infant (IQR 16 – 21, range 6 – 24). These infants had a median of eight serotype acquisitions (IQR 7 – 10, range 2 – 15) in the first two years of life. To assess whether the length of time an infant carried a pneumococcus was associated with changes in specific IgG titre serum anti-protein IgG fold-changes from 6 – 24 months of age (i.e. following the loss of maternally-derived antibody) were compared with the number of months in which pneumococci were identified in the nasopharynx over the first two years of life. Duration of colonisation was not associated with an increased IgG but for anti-PiaA and PiuA IgG, titres were negatively correlated (Table 32). As an alternative measure of pneumococcal exposure, the total number of pneumococcal serotype acquisitions was compared with antibody responses and a small positive correlation was found for PspA-Fam2 specific IgG. Restricting both analyses to colonisation in the second year of life, i.e. when the immune system is more mature, antibody titres to four proteins (LytC, PhtD-1, PhtE, and SP2027)

were weakly positively correlated with the number of months in which pneumococci were identified in the nasopharynx and titres to PhtD (both PhtD-1 and -2) were positively correlated with number of new pneumococcal acquisitions over this time (Table 32).

To determine whether serum IgG anti-protein antibodies were protective against colonisation, twelve month antibody titres were compared with the number of new pneumococcal acquisitions occurring in the second year of life but no protection was demonstrated. In contrast, IgG titres specific for three proteins were weakly correlated with an increased risk of carriage; Ply (Ply-2; $r = 0.21$, 95% CI 0.02 – 0.40), PspA-Fam1 ($r = 0.29$, 95% CI 0.10 – 0.47), and StkP ($r = 0.20$, 95% CI 0.03 – 0.41).

Table 32. Associations between serum IgG antibody titre and pneumococcal acquisitions or colonisation duration in infants

For clarity, only statistically significant results ($P < .05$) are shown.

Protein	Colonisation duration		Acquisitions	
	6-24m IgG titre fold-change and colonisation 0-24m[a]	12-24m IgG titre fold-change and colonisation 13-24m[a]	6-24m IgG titre fold change and acquisitions 0-24m[a]	12-24m IgG titre fold change and acquisitions 13-24m[a]
LytC		0.22 (0.01 – 0.40)		
PhtD-1				0.22 (0.02 – 0.41)
PhtD-2		0.23 (0.03 – 0.42)		0.21 (0.01 – 0.40)
PhtE		0.21 (0.01 – 0.40)		
PiaA	-0.27 (-0.41 – -0.02)			
PiuA	-0.40 (-0.56 – -0.21)			
PspA-Fam2			0.22 (0.02 – 0.41)	
SP2027		0.25 (0.04 – 0.43)		

[a] Pearson correlation coefficients (with 95% confidence intervals) for fold change in serum IgG antibody titre between either 6 or 12 months and 24 months versus number of months colonised by, or new acquisitions of, *S. pneumoniae* in either the first two years of life or just the second year

5.2.6. Discussion

This study has documented the development of serum IgG to a large panel of pneumococcal surface and virulence proteins, and the effect of transplacental transfer of

these antibodies, in a population of SE Asian infants where pneumococcal colonisation occurs early in life and is sustained until at least 24 months of age.

Serum IgG antibodies to all proteins were detected in mothers sampled at delivery. Antibody titres to LytB, PcpA, PhtD, and PhtE were significantly higher in mothers who were concurrently colonised by pneumococci at the time of delivery, indicating that these proteins are immunogenic in adults. However, they did not appear to protect their infants from early colonisation. While higher cord blood IgG titres to PiuA and Spr0096 were associated with a delay in infant pneumococcal colonisation in univariate analyses, the association was lost in a multivariate Cox model where only environmental factors remained a significant influence on the timing of initial infant colonisation. Previous studies in young infants from the Philippines and Papua New Guinea concluded that maternally derived anti-pneumolysin antibodies were associated with delayed infant colonisation (71, 111). The Papua New Guinean study also found that higher maternal anti-PspA family 1 antibodies were associated with earlier colonisation. Reproducing the logistic regression analysis described in the Philippine study did demonstrate a trend towards delayed colonisation with higher anti-Ply titres in the Maela cohort (data not shown), but the Cox regression model remained a more appropriate analysis for this dataset. Differences in environmental factors (and the fact that these were included in the Cox model in the current study), nasopharyngeal sampling frequency, or assay methodology may account for the differences between the Maela results and those from the Philippines.

Infants began developing antibodies to selected proteins from two months of age, and this was largely in response to nasopharyngeal colonisation. Certain proteins were clearly more immunogenic than others in young infants. For example, antibodies to PsaA rose rapidly from two months and the GMT at 12 months was greater than the cord blood GMT. This protein has previously been shown to be highly immunogenic in studies from Kenya and the Philippines (111, 211). Responses to other proteins were more modest

under one year of age, but infants had considerable antibody responses in the second year of life (e.g. PhtD and PhtE). A small number of proteins, notably CbpA, StkP, and PspA were poorly immunogenic throughout the study period. Although antibodies to pneumococcal pilus proteins are highly protective against invasive disease, antibody responses to pilus proteins RrgA, RrgB, and RrgC were relatively modest following colonisation, and this likely reflects the non-conserved nature of the pilus-encoding genes amongst pneumococci (224). Indeed, the PI-1 prevalence was only 35.2% in pneumococci carried by the Maela cohort (section 6.1).

The relationship between serum IgG titres at 12 months and the risk of carriage during the second year of life was assessed and there was no protective effect found for antibodies to any of the proteins, in terms of a reduction in the number of new pneumococcal acquisitions. Indeed, for Ply (Ply-2), PspA-Fam1 and StkP, the opposite was true: higher IgG titres at 12 months were associated with a greater number of nasopharyngeal acquisitions in the second year of life. This might indicate relatively greater pneumococcal exposure, as a result of household or community contact, in these infants which would explain higher antibody titres at 12 months of age. This conclusion fits with the observation that, for several proteins, fold-changes in antibody titres between six or 12 and 24 months were positively correlated with either the number of new pneumococcal acquisitions or the total number of months an infant was colonised. An alternative explanation for the positive correlation between 12 month IgG titres and pneumococcal acquisitions in the second year of life is that these antibodies, or mucosal IgA1 antibodies to the same protein(s), facilitate pneumococcal colonisation, perhaps by enhancing attachment to the nasopharyngeal mucosa as has been demonstrated previously for anti-capsular IgA1 antibodies (320). The absence of a protective effect of antibody on colonisation may reflect the likely high level of exposure to pneumococci in the community in this population but has also been documented for children in Finland and the Netherlands and in adults in the UK (184, 200, 203, 206). These finding were in also

agreement with results of a mouse colonisation model which concluded that serum antibodies to PsaA, PspA, or PpmA (putative proteinase maturation protein A) were not responsible for protection against nasopharyngeal colonisation (321). However, there is evidence that this may not be the case for all human age groups: colonised children aged 2 – 12 years in the UK had lower CbpA and Ply antibody titres than non-colonised children, indicating possible protective effects (208). Also, PspA has previously been shown to protect against experimental colonisation of healthy North American adults (212). Of course, these results do not necessarily predict the protective quality of the immune response elicited by active immunisation against any of the antigens, although the negative correlation between antibody responses to PiaA and PiuA and number of swabs positive for pneumococcus in the first two years of life suggests that tolerance and hyporesponsiveness may occur if these proteins were used as components of a pneumococcal vaccine.

The early acquisition and persistence of pneumococcal colonisation in the nasopharynx of the study infants were both the most important positive and negative aspects of this study. After three months of age more than two-thirds of infants were colonised at each monthly time point, permitting determination of the kinetics of serum IgG development to pneumococcal proteins in a population almost continually exposed to *S. pneumoniae*. In terms of pneumococcal colonisation, this population is similar to many developing world settings where effective pneumococcal vaccines with broad coverage (i.e. not restricted to 7 – 13 serotypes) will have the most benefit. The large panel of protein antigens studied and inclusion of serum samples from the second year of life, along with associated colonisation data, builds on the findings of the work from Papua New Guinea and the Philippines, the two cohorts most similar to the Maela cohort in terms of pneumococcal exposure (71, 111, 210). However, this high level of colonisation, coupled with the monthly swabbing interval, limited the ability to detect small differences in the timing of first pneumococcal acquisition in relation to cord blood antibody titres. In Papua

New Guinea weekly swabs were taken from infants during the first month of life, resulting in a higher resolution than achieved in the Maela study (71). Detection of correlation between antibody titres and colonisation duration or pneumococcal acquisitions were also not completely ideal in the current study, since the monthly swabbing interval and the imperfect sensitivity of the culture technique may have resulted in missed pneumococcal carriage episodes.

In conclusion, this study demonstrates clearly that there is considerable variation in the immunogenicity of pneumococcal surface and virulence proteins in infancy. However, nasopharyngeal colonisation by *S. pneumoniae* in the first two years of life results in demonstrable serum IgG responses to these proteins but these antibodies do not provide protection against subsequent colonisation in an environment where there is frequent exposure to pneumococci.

6 Potential modifiers of pneumococcal colonisation: the pneumococcal pilus

6.1. Assessment of *Streptococcus pneumoniae* PI-1 prevalence in carried and transmitted isolates from mother-infant pairs on the Thailand-Burma border

6.1.1. Summary

Background

Streptococcus pneumoniae PI-1 encoded pilus enhances *in vitro* adhesion to the respiratory epithelium and may contribute to pneumococcal nasopharyngeal colonisation and transmission. The pilus subunits are regarded as potential protein vaccine candidates. The aim of this study was to determine PI-1 prevalence in carried pneumococcal isolates and explore its relationship to transmissibility or carriage duration.

Methods

Eight hundred and ninety six pneumococcal carriage isolates collected from mother-infant pairs during the Maela cohort study were selected for analysis. These isolates were chosen according to specific carriage and transmission definitions and PI-1 PCR and genotyping by Multi Locus Sequence Typing was done on all.

Results

Overall 35.2% of the isolates were PI-1 positive, but PI-1 presence was restricted to 10 of the 34 serotypes studied and most frequently associated with serotypes 19F and 23F. 47.5% of transmitted and 43.3% of non-transmitted pneumococci were PI-1 positive (OR 1.2; 95% CI: 0.8 – 1.7; $P = .4$). The duration of first ever infant pneumococcal carriage was significantly longer with PI-1 positive organisms, but this difference was not significant at the individual serotype level.

Conclusions

PI-1 is commonly found in pneumococcal carriage isolates but does not appear to be associated with pneumococcal transmissibility or carriage duration.

6.1.2. Introduction

The pneumococcal pilus-1 is encoded by the pilus islet-1 (PI-1; *rlrA* islet) and is composed of three subunits, RrgA, RrgB, and RrgC (219-221). The RrgA subunit of the surface exposed pilus-1 filamentous structure has been shown to enhance pneumococcal adherence to respiratory epithelial cells *in vitro* (218, 219). The impact of this finding has not been assessed in human carriage studies to date.

Immunisation with pilus antigens is protective against lethal intraperitoneal challenge in a mouse model and therefore pilus subunits are regarded as potential candidates for inclusion in a protein-based pneumococcal vaccine (222). However, PI-1 presence is not universal: studies of predominantly invasive pneumococci have found that PI-1 is present in isolates from a limited number of serotypes, particularly those included in the 7-valent conjugate vaccine, and that its presence correlates with MLST genotype (8, 197, 223-226). Interestingly, recent data from Massachusetts suggests that pilus presence is advantageous to pneumococci as evidenced by its increasing prevalence in non-vaccine serotypes following PCV introduction (322).

6.1.3. Aims and objectives

The aims of the current study were to: i) determine PI-1 prevalence in pneumococcal carriage isolates from infants and their mothers; ii) determine which PI-1 positive clones were associated with carriage in the study region; iii) explore the possible functions of the pilus *in vivo*, and in particular evaluate whether PI-1 presence had an effect on pneumococcal transmission or carriage duration.

6.1.4. Methods

6.1.4.1. Pneumococcal isolates

Pneumococcal isolates cultured from NPS specimens collected from the "immunology" follow-up group (section 2.2.2) of the pneumonia cohort were included in this study. Mother and infant pneumococcal carriage patterns for the first twelve months of follow-up were reviewed and isolates chosen for further analysis using the definitions described below.

6.1.4.2. Definition of transmission and carriage

Transmission of a pneumococcus was defined as the presence of an identical pneumococcal serotype and MLST genotype in both mother and infant at the same visit ("concordant transmission") or when isolated from the mother and the infant during a "transmissibility episode" but not at the same visit ("discordant transmission"). A non-transmitted pneumococcus was a carried serotype that never appeared in the other member of the mother-infant pair during the "transmissibility episode" as defined below (Figure 29). "Transmissibility episodes" were identified by carriage within a mother-infant pair where there was the same pneumococcal serotype cultured from $\geq$2 NPS (from mother and/or infant), separated by $\leq$2 NPS negative for that serotype. For serotypes 1 and 5, known to be carried for very short durations, a single positive NPS could define non-transmission.

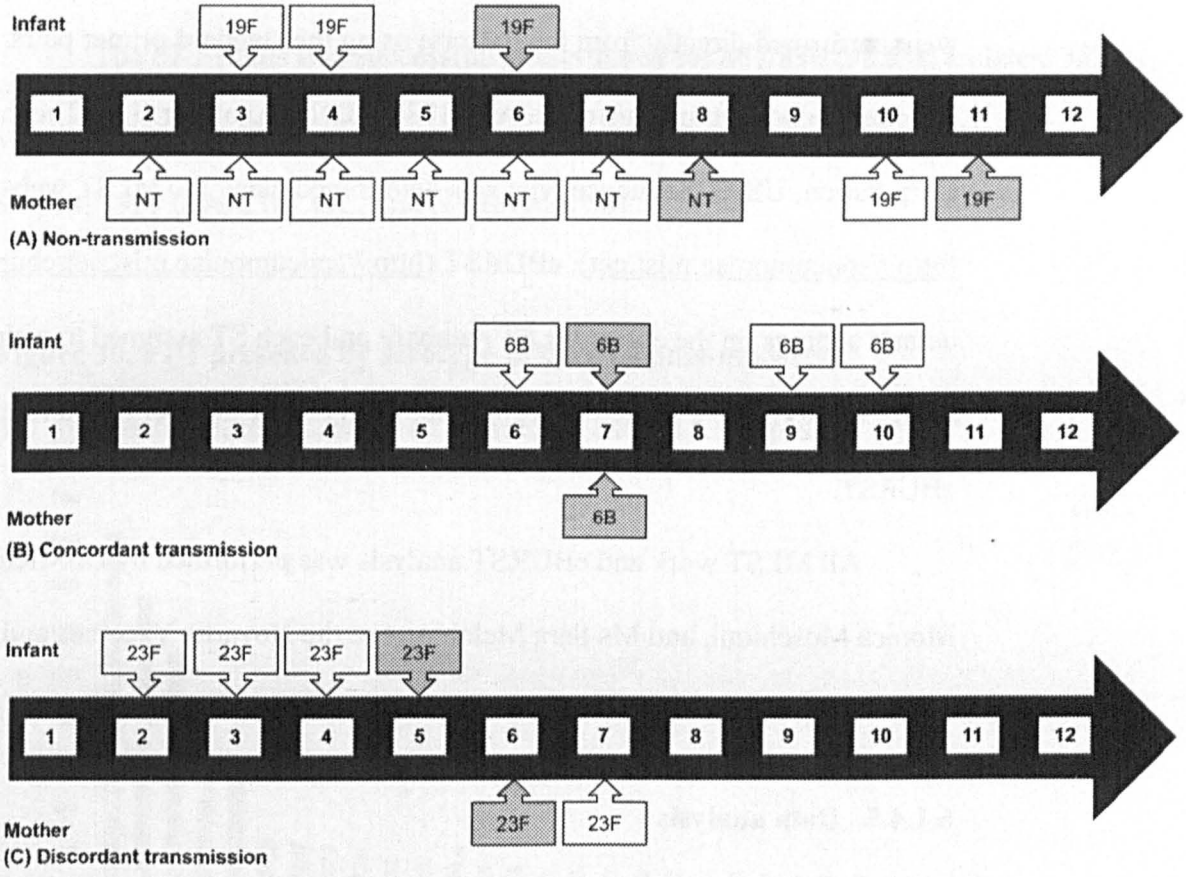
For the determination of carriage duration in infants, a pneumococcal acquisition was defined as midpoint between the last negative swab and the first positive swab for a given serotype. Termination of the carriage episode was similarly defined as the midpoint between the first of two consecutive negative swabs and the last positive swab for the serotype.

Figure 29. Pneumococcal serotype transmission examples

(A) Non-transmission of serotypes 19F and NT;

(B) Concordant transmission of serotype 6B;

(C) Discordant transmission of serotype 23F. Horizontal arrows represent an infant's first year of life, with the numbers indicating age in months. Pneumococcal serotypes shown above these arrows represent carried isolates from the infant and those below the arrows are from the mother. Shading highlights the criteria used to select isolates for PI-1 PCR and MLST.



6.1.4.3. PI-1 detection

PI-1 presence was determined as previously described (224). Briefly, PCRs were performed directly from bacteria using the primers listed in Table 33. Primers were designed on conserved regions on the boundaries of PI-1 (459for, 470rev) and within PI-1 (P01rev, P11for, P08for, P08rev).

Table 33. PI-1 PCR primer sets

Forward primer	Sequence (5'→3')	Reverse primer	Sequence (5'→3')
459 for	AACTGAATTGACACAACGTGTCTT	470 rev	GCCACACAAGATGTTGATGCTTTT
459 for	AACTGAATTGACACAACGTGTCTT	P01 rev	AGCGACAAGCCACTGTATCATATT
P08 for	TGAGATTTTCTCGTTTCTCTTAGC	P08 rev	AATAGACGATGGGTATTGATCATGT
P11 for	GCCATTTGGATCAGCTAAAAGTT	470 rev	GCCACACAAGATGTTGATGCTTTT

6.1.4.4. Multi Locus Sequence Typing

MLST was performed as previously described (30). Briefly, PCR amplifications were performed directly from the bacteria using the standard primer pairs. Sequences were obtained on both strands using an ABI 3730xl DNA Analyser (Life Technologies Corporation, USA). Sequence type was determined using the MLST website (http://spneumoniae.mlst.net). eBURST (http://spneumoniae.mlst.net/eburst/) was run with default settings on the entire MLST database and each ST assigned to a clonal complex (CC) (31). CCs were named in accordance with the ST number of the founder predicted by eBURST.

All MLST work and eBURST analysis was performed by Dr Michele Barocchi, Dr Monica Moschioni, and Ms Sara Melchiorre at the Novartis Vaccines and Diagnostics laboratories, Siena, Italy.

6.1.4.5. Data analysis

Chi-squared test, Fisher's exact test, and odds ratios were used to compare proportions. Carriage duration was estimated by survival analysis methods, using the log-rank test to compare groups (73).

6.1.5. Results

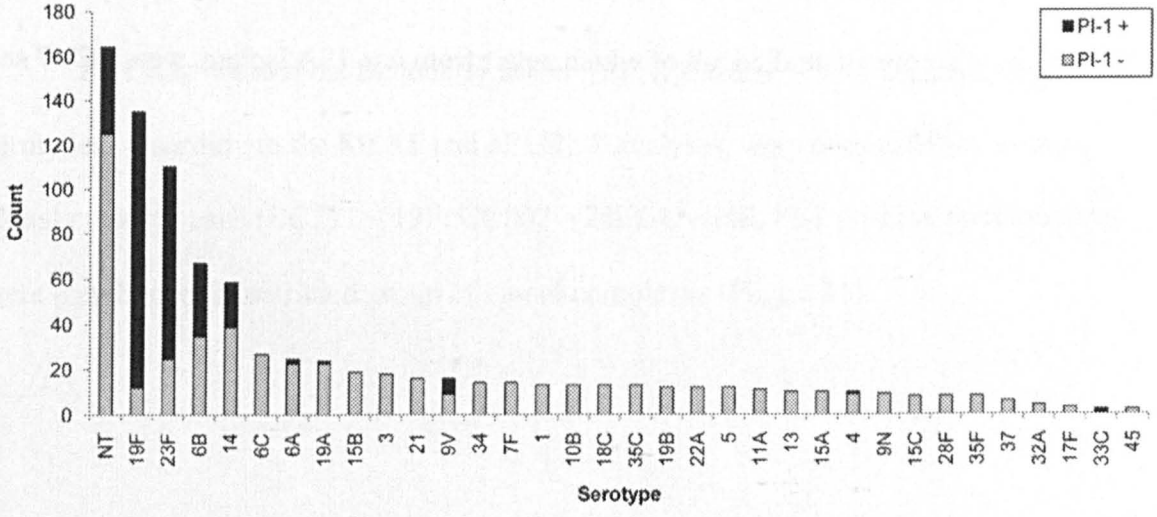
6.1.5.1. Pneumococcal isolates and PI-1 prevalence

In the first twelve months of follow-up, 4,921 surveillance NPS were collected from the 234 mother-infant pairs (84.7% of expected). 2,497 pneumococci were cultured and 896 (35.9%) of these isolates were included in the current work. All pneumococcal isolates meeting the criteria described below were included, resulting in thirty-four serotypes being represented in the strain selection. These serotypes accounted for 90.1% (2,251/2,497) of isolates cultured from swabs collected in the first year of follow-up.

The PI-1 status was successfully determined for 887/896 (98.9%) isolates: 35.2% (312/887) were positive. PI-1 positive isolates were restricted to 10 serotypes: 4, 6A, 6B, 9V, 14, 19F, 19A, 23F, 33C, and NT (Figure 30).

Figure 30. PI-1 presence by serotype in carried pneumococci

887 pneumococci, representing all commonly carried serotypes.



6.1.5.2. PI-1 and transmission

To analyse whether PI-1 presence had an effect on pneumococcal transmission within the mother-infant pairs, a panel of strains was selected and analysed by both PI-1

PCR and MLST (to define genotype). The serotypes selected for study were those contained in, or related to, current conjugate vaccines (PCV13 + 6C) as well as non-typeable pneumococci which were overrepresented in the study population (the commonest pneumococcal "type" identified from mother swabs and the third commonest in infants). In the first year of follow-up, these serotypes accounted for 69.6% and 59.8% of pneumococci carried by infants and mothers, respectively. In the case of a potentially transmitted pneumococcal serotype, i.e. isolates carried by both mother and infant at the same visit, or at sequential visits, single isolates from both mother and infant were selected for study. For non-transmitted pneumococci, i.e. pneumococci carried by only the mother or infant, a single isolate was studied. Additionally, every isolate from carriage episodes of all serotypes in eight mother-infant pairs was analysed to determine the clonality of pneumococcal carriage.

Of the 489 isolates selected for the primary analysis, PI-1 PCR was uninterpretable in six, resulting in 483 analysable pneumococcal isolates. Overall, 219/483 (45.3%) of these isolates were PI-1 positive. PI-1 was found in non-typeable pneumococci and in 6/14 serotypes studied, all of which, apart from one 19A isolate, were PCV7 serotypes (Table 34).

Table 34. Pneumococcal serotype distribution, pilus presence, and isolate transmission category in mother-infant pairs

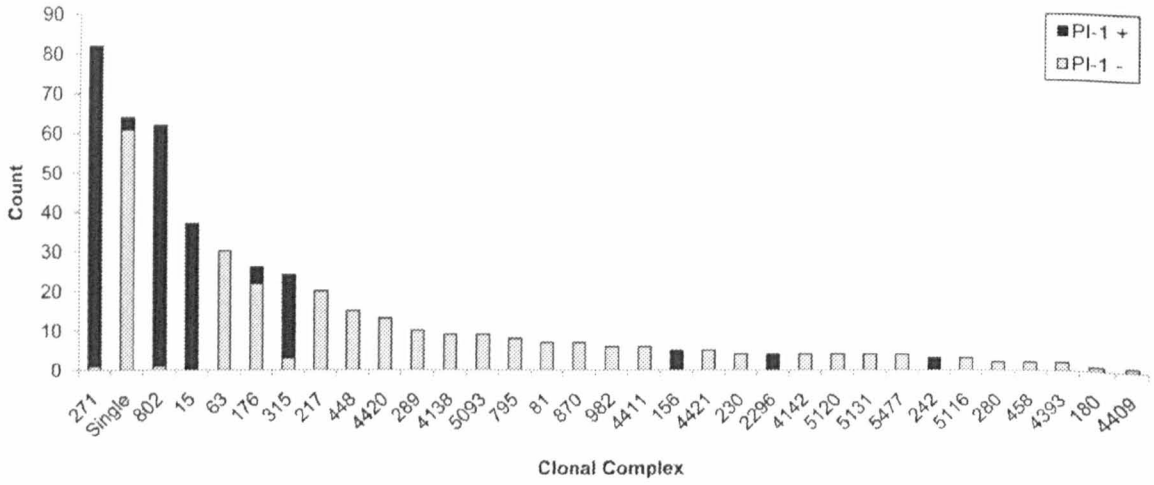
Serotype	Total, N	Transmitted[a], N (% within serotype)	PI-1 present, N (% within serotype)
1	12	4 (33.3)	0
3	11	4 (36.4)	0
4	4	2 (50.0)	0
5	10	2 (20.0)	0
6A	16	6 (37.5)	0
6B	54	27 (50.0)	29 (53.7)
6C	15	4 (26.7)	0
7F	5	2 (40.0)	0
9V	8	4 (50.0)	5 (62.5)
14	46	28 (60.9)	15 (32.6)
18C	7	4 (57.1)	0
19F	89	50 (56.2)	81 (91.0)
19A	14	4 (28.6)	1 (7.1)
23F	82	38 (46.3)	63 (76.8)
NT	110	57 (51.8)	25 (22.7)
Total	483	236 (48.9)	219 (45.3)

[a] Concordant and discordant transmission combined

PI-1 was found most frequently in 19F (91%) and 23F (77%) although these serotypes, according to the MLST and eBURST analyses, were dominated by a single clonal complex each (CC271 – 19F; CC802 – 23F). Overall, PI-1 positive pneumococci were members of a restricted group of clonal complexes (Figure 31).

Figure 31. PI-1 presence by clonal complex

483 carried pneumococci (PCV13 serotypes plus 6C and NT).



By serotyping a representative isolate alone, 253/483 (52.4%) pneumococci would have been classified as transmitted (153 concordant; 100 discordant) and 230/483 (47.6%) as non-transmitted. Combining MLST and serotype, nine concordant and eight discordant "transmitted" isolates (17/483; 3.5%) were reclassified as non-transmitted, resulting in a final total of 236/483 (48.9%) transmitted and 247/483 (51.1%) non-transmitted pneumococci. PI-1 presence was not correlated with transmission: 47.5% (112/236) of transmitted and 43.3% (107/247) of non-transmitted pneumococci were PI-1 positive, with an odds ratio of 1.2 (95% CI 0.8 – 1.7, $P = .4$), and no significant differences at the individual serotype level.

An additional 91 pneumococcal isolates, from seven mother-infant pairs and one mother-twin infant unit, were analysed to confirm that sequential isolates of the same serotype within an individual, and isolates of the same serotype within a mother-infant pair, were indeed identical. In all mother-infant pairs each serotype carried was represented by a single ST per carriage/transmission episode with three exceptions (Table 35).

Table 35. Detailed study of pneumococcal carriage in the first year of life in eight mother-infant pairs

Pneumococcal serotypes and their MLST genotype (in parentheses) are given for each nasopharyngeal sampling point.

Pair	Category	Infant age (m)											
		1	2	3	4	5	6	7	8	9	10	11	12
Pair 1	Infant			19A (230)	19A (230)	14 (63)	14 (63)	14 (63)	NT (4133)	6C (4420)	6C (4420) NT (4133)	6C (4420)	6C (4420)
	Mother			35F (4418)	14 (63)							7F (3545)	7F (3545)
Pair 2	Infant		NT (4136)	NT (4136)	NT (4136)		NT (4136)	NT (4136)	NT (4136)	NT (4136)	NT (4136)		
	Mother	NT (4136)		7F (3545)	7F (3545)	NT (4136)	7F (3545)	7F (3545)	7F (3545) NT (4136)	7F (3545)	NT (4136)	NT (4136)	NT (4136)
Pair 3	Infant		14 (63)	14 (63)	14 (63)	NT (4451)	14 (63)	14 (63)		19F (81)	22A (910)	19F (81)	22A (910)
	Mother	32A (5092)	32A (5092)	32A (5092)	NT (5121)	14 (63)	NT (4451)	14 (4451)	NT (4451)	32A (5092)		NT (4451)	19F (81)
Pair 4	Infant			19F (4414)	19F (4414)	19F (4414)	19F (4414)	19F (4414)	19F (4414)	23F (802)	19F (4414)	19F (4414)	23F (802)
	Mother	37 (447)	37 (447)			37 (447)	37 (447)		37 (447)	37 (447)			19F (4414)
Pair 5	Infant 1	NT (448)	33C (5096)	33C (5096)	17F (5098)	21 (5103)	21 (5103)	19F (5106)	19F (5106)	19F (4414)	19F (4414)	6A (4936)	19F (4414) NT (448)
(twins)	Infant 2		17F (5098)	21 (5103)	21 (5103)	17F (5098)	21 (5103)	21 (5103)	21 (5103)		19F (4414)	6A (4936)	6A (4936)
	Mother	NT (448)		1 (217)			NT (448)						
Pair 6	Infant		NT (4133)	NT (4133)	19B (5095)	1 (217)	NT (4133)	9V (280)	9V (280)	19F (4414)	19F (4414)	19F (4414)	19F (4414)
	Mother	19B (5095)	19B (5095)			NT (4133)			9V (5123)	9V (280)			
Pair 7	Infant	45 (5097)	45 (5097)	23F (802)	23F (802)	23F (802)		6B (315)		23F (802)	23F (802)	23F (802)	
	Mother		23F (802)		23F (802)	23F (802)	6B (315)					23F (802)	
Pair 8	Infant			23F (4413)			23F (4413)	23F (4413)	23F (4413)	23F (4413)		23F (4413)	
	Mother					23F (4413)						23F (4413)	

6.1.5.3. PI-1 and first pneumococcal carriage episode duration in infants

To analyse the correlation between PI-1 presence and the duration of infant carriage, the PI-1 status was determined for 316 isolates from first carriage episodes of all common serotypes (defined as serotypes with at least ten carriage episodes). These episodes included first episodes of carriage of each serotype, not only an infant's first ever pneumococcal carriage episode. PI-1 status for the entire carriage episode was inferred from the PI-1 PCR result of a single isolate.

Given the well described association of shorter carriage in individuals with prior pneumococcal exposure, analysis was subsequently focussed on only first ever episodes of carriage (i.e. one per infant) (73). From the 316 carriage episodes selected, 216 analysable first ever carriage episodes were identified: PI-1 PCR data were available for at least one isolate in 180 of these (90 PCV13 serotypes, 58 non-vaccine serotypes, and 32 non-typeable pneumococci). Median and mean durations of these first pneumococcal carriage episodes were 63 days (95% CI 61 – 91) and 105 days (95% CI 91 – 119) respectively, but varied considerably by serotype as previously described (Table 36).

Table 36. First pneumococcal carriage episode duration for the six commonest serotypes

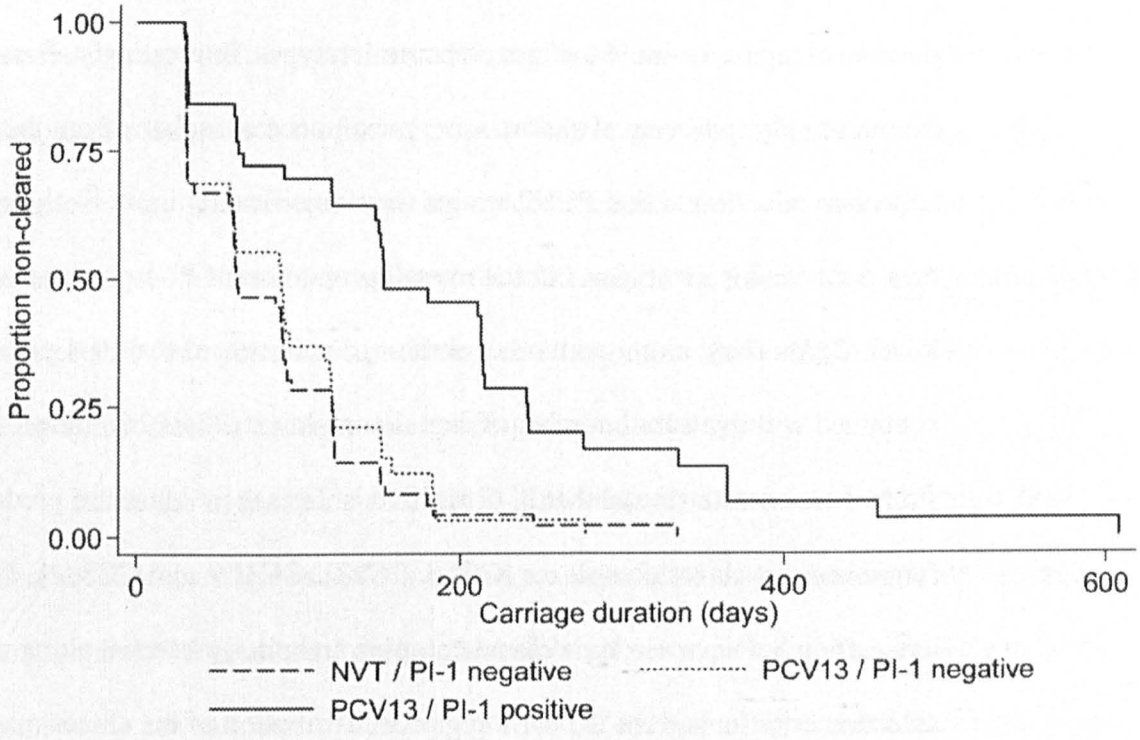
Serotype	Number of episodes	Carriage duration (days), median (95% CI)
6B	13	121 (90 – 153)
14	8	62 (30 – 151)
19F	21	213 (63 – 243)
23F	21	184 (62 – 277)
35F	9	121 (30 – 180)
NT	33	31 (31 – 61)

Comparing the 180 carriage episodes where PI-1 PCR results were available, PI-1 positive carriage episodes (51/180; 28.3%) were significantly longer than those associated

with a PI-1 negative organism (median 152 days (95% CI 93 – 213) vs. 61 days (95% CI 57 – 90); $P < .0001$) (Figure 32). However, the analysis of carriage duration was confounded by serotype. Serotypes 19F and 23F had the longest duration of carriage and were both predominantly PI-1 positive. To analyse the relative contribution of serotype and PI-1 to carriage duration a Cox regression model was fitted: stratifying carriage by serotype, PI-1 presence was not associated with a significant change in carriage duration (HR 0.7 (95% CI 0.4 – 1.2; $P = .2$)).

Figure 32. Impact of pilus on first pneumococcal carriage episode duration in infants

Stratified by vaccine or non-vaccine serotype and PI-1 presence. All NVT isolates were PI-1 negative.



6.1.6. Discussion

This study is the largest investigation of the prevalence and possible function of the PI-1 encoded pilus in carriage isolates of *S. pneumoniae* reported in the literature to date.

As serotypes are commonly used as the basis for defining transmission, it was of interest to determine in the frequency with which a molecular analysis may confound the analysis of transmission. By genotyping, discrepancies were discovered in only 3.5% of transmission events classified by serotype. The study also demonstrated that pneumococci of the same serotype are predominantly clonal within a discrete carriage episode. It can therefore be concluded that serotype can be used to define transmission events and carriage episodes with relatively high confidence, but the inclusion of genotyping is vital to ensure complete accuracy.

Because of the selection criteria for isolates included in the study, the overall pilus prevalence for the population cannot be described. However, in the 887 carriage isolates analysed the pilus prevalence was 35.2%. Of the 34 serotypes analysed, PI-1was only present in non-typeable isolates and nine other serotypes: 65% of PCV7 isolates were PI-1 positive compared with 9% of non-vaccine serotypes. Interestingly, Basset et al., examining nasopharyngeal and invasive pneumococcal isolates from the American Indian collection, also found that PCV7 strains were significantly more likely to be PI-1 positive than non-vaccine serotypes, but the overall proportion of PI-1 positive isolates was slightly lower (223). They, along with other authors, demonstrated that PI-1 positive strains were contained within a small number of clonal complexes (224-226). Indeed, in the current study it was demonstrated that PI-1 positive isolates were clustered predominantly within four dominant clonal complexes (CC15, CC271, CC315, and CC802). The strong association between serotype/clonal complex and pilus presence, along with strain selection criteria, and the different regional distribution of the clones may explain the variability in PI-1 prevalence between different studies.

A pilus-attributable effect on pneumococcal transmissibility could not be demonstrated. However, there are limitations to the study which may be important confounders for this analysis. The carriage study was carried out in a densely populated refugee camp where 15% of the population are <5 years old and there is likely to be

frequent transmission of nasopharyngeal organisms both within families and between members of the community. Despite using a combination of serotype and sequence type to increase the accuracy of the transmissibility categories, the possibility that "non-transmitted" strains were effectively transmitted between mother or infant and others but were not detected cannot be excluded. Several studies have documented the clustering of pneumococcal serotypes and genotypes within families, which highlights the difficulty of assigning a definitive "non-transmitted" label to isolates collected from an incomplete household group (170, 176). However, the study focused on mother-infant transmission since it was felt that the absence of a strain in one member of this pair would be the best marker for relative non-transmissibility in the early months of life. In addition, both concordant and discordant time-point pneumococcal serotype/ST identifications in the mother-infant pair were included as "transmitted", as long as they occurred no more than two months apart. However, the study sampling frequency may have been too low to demonstrate transmission of serotypes carried for very short durations. Also, in the presence of multiple serotype carriage, a common occurrence in infancy and one that is underestimated by standard culture protocols (section 4), a particular serotype may become undetectable for a period of time before re-emerging as the dominant serotype which may result in incorrect categorisation regarding transmissibility.

Although in the crude analysis PI-1 presence was associated with longer first pneumococcal carriage episode in infants, no significant association between carriage duration and PI-1 could be found at the individual serotype level. This is likely to be the result of the low numbers of carriage episodes of individual serotypes (resulting in wide confidence intervals around the carriage duration estimates) and the restricted number of clones within each serotype. The number of carriage episodes analysed could have been increased by looking at all carriage episodes of each serotype rather than restricting to each infants first ever carriage episodes, but this would have introduced other confounding factors, such as the impact of previous carriage and immune factors in subsequent carriage

episode duration. Therefore, it is possible that, as a result the study sample size, a small effect of pilus on either transmission or carriage duration may have been missed.

In conclusion, SE Asian pneumococcal carriage isolates had similar pilus prevalence to previously described strain collections, which is helpful in the on-going assessment of likely global coverage of a pilus subunit-containing vaccine. Despite its known role in pneumococcal attachment, an unequivocal impact of PI-1 presence on transmissibility or carriage duration could not be determined.

7 Potential modifiers of pneumococcal colonisation: other inhabitants of the nasopharynx

7.1. The relationships between non-pneumococcal bacterial colonisation, viral infection and pneumococcal nasopharyngeal colonisation

7.1.1. Summary

Background

Interactions between the various colonisers of the nasopharyngeal space are complex and incompletely understood. The aim of this study was determine the impact of co-colonisation by bacteria and viruses on pneumococcal colonisation in infants and their mothers.

Methods

Nasopharyngeal swabs collected from infants during the Maela cohort study were cultured onto both CNA-blood and chocolate agar plates to detect bacterial colonisers, in particular *H. influenzae, M. catarrhalis*, *S. aureus,* and *S. pneumoniae.* The impact of viral infection, detected by PCR of nasopharyngeal aspirates during pneumonia episodes, on bacterial colonisation was also assessed in these infants. Maternal swabs collected at six-monthly intervals were re-cultured to determine interactions between *S. pneumoniae* and *S. aureus.*

Results

Staphylococcus aureus was acquired at an early age (median 17 days) but only 65.8% were colonised during the first 24 months of life. *H. influenzae* and *M. catarrhalis* were acquired slightly later (median 46 and 61 days, respectively) and colonisation was almost universal. In a multivariate model, recent antimicrobial use (OR 0.4, $P < .001$) and *S. aureus* colonisation (OR 0.3, $P < .001$) were negatively associated with pneumococcal colonisation, whereas *H. influenzae* (OR 2.9, $P < .001$) or *M. catarrhalis* (OR 2.1, $P < .001$) colonisation were positively associated. During first pneumonia episodes, detection

of respiratory syncytial virus was negatively associated with pneumococcal colonisation (OR 0.5, $P = .02$). Although a trend to a negative association between *S. pneumoniae* and *S. aureus* colonisation in mothers was found in a crude analysis, this was lost when controlling for clustering (OR 0.7, $P = .3$).

Conclusions

Co-colonisation of the nasopharynx by several bacterial pathogens was common, and occurred early, in Maela infants. The observed negative association between RSV infection and pneumococcal colonisation requires further study.

7.1.2. Introduction

Bacterial colonisation of the nasopharynx is a complex dynamic and interactive process. Previous studies of children from geographically diverse locations have generally documented positive correlations between nasopharyngeal colonisation by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* (105, 235, 241-243) and a negative correlation between *S. aureus* and *S. pneumoniae* (48, 233, 234). Viral infection of the respiratory tract predisposes to bacterial colonisation by several mechanisms which reduce clearance or increase adherence of bacteria to the respiratory epithelium (256, 257). Few studies of colonisation have specifically focussed on the interactions between both bacteria and viruses (241, 245).

7.1.3. Aims and objectives

The aim of this study was to explore the relationships between both common bacterial and viral colonisers of the nasopharynx.

7.1.4. Methods

Three selections were made from specimens collected during the Maela cohort study.

7.1.4.1. Associations between bacterial nasopharyngeal colonisers in infants

Nasopharyngeal swab specimens collected from the "immunology" follow-up group of the Maela pneumonia cohort were further analysed.

Ten microlitres of thawed STGG from all 4,191 infant NPS were cultured onto a chocolate agar plate (Clinical Diagnostics, Bangkok, Thailand) at the same time as 10 µL STGG was cultured onto CNA-blood agar (bioMerieux, Marcy L'Etoile, France) to detect *S. pneumoniae* colonisation as previously described (section 2.2.5.1.2). After overnight incubation at 35-37°C in 5% CO_2, plates were examined for the presence of target organisms (*H. influenzae* and *M. catarrhalis*). Colonisation by *S. aureus* was established by examination of the CNA-blood agar plate. Target organisms were confirmed by standard microbiological techniques as described in section 2.2.5.1.3. Briefly, *H. influenzae* was confirmed by X+V factor dependent growth. All isolates of *H. influenzae* were serotyped by slide agglutination using polyclonal and monoclonal antisera (Becton Dickinson, Franklin Lakes NJ, USA). *M. catarrhalis* was confirmed by colony morphology and trybutyrin production (Rosco Diatabs, Taastrup, Denmark). *S. aureus* was identified by DNase production and the slide coagulase test.

7.1.4.2. Associations between *Staphylococcus aureus* and *Streptococcus pneumoniae* colonisation in mothers

To further explore the potential interactions between *S. pneumoniae* and *S. aureus* colonisation, nasopharyngeal swabs from mothers who had provided swabs at the delivery, 6 month, 12 month, 18 month, and 24 month visits were thawed and 10 µL of STGG was re-cultured onto CNA-blood agar plates. *S. aureus* was identified as described previously.

7.1.4.3. Associations between bacterial nasopharyngeal colonisers and viral infection

All cohort infants (i.e. "immunology" and "routine" follow-up groups combined) had a nasopharyngeal swab and aspirate collected if they were brought to the clinic unwell and met the WHO clinical case definition for pneumonia (section 2.2.3.3). The NPS were

cultured on CNA-blood agar and chocolate agar plates as previously described (section 2.2.5.1). For "routine" follow-up infants, whose monthly surveillance NPS were not automatically cultured, NPS from the 2 – 3 months preceding the pneumonia episode NPS were also cultured. Respiratory viruses were detected in NPA specimens as described in section 2.2.5.3.

7.1.4.4. Data analysis

Times to acquisition of target pathogens were estimated by survival analysis. For longitudinal data, associations between organisms were assessed by generalised estimating equations with a logistic link and exchangeable correlation structure to account for multiple observations per individual. For the pneumonia episodes, organism associations were assessed by multivariate logistic regression. Proportions were compared by chi-squared test. The organism heat map was generated in Stata/IC 12.1 (StataCorp, College Station TX, USA) using the "hmap" command.

7.1.5. Results

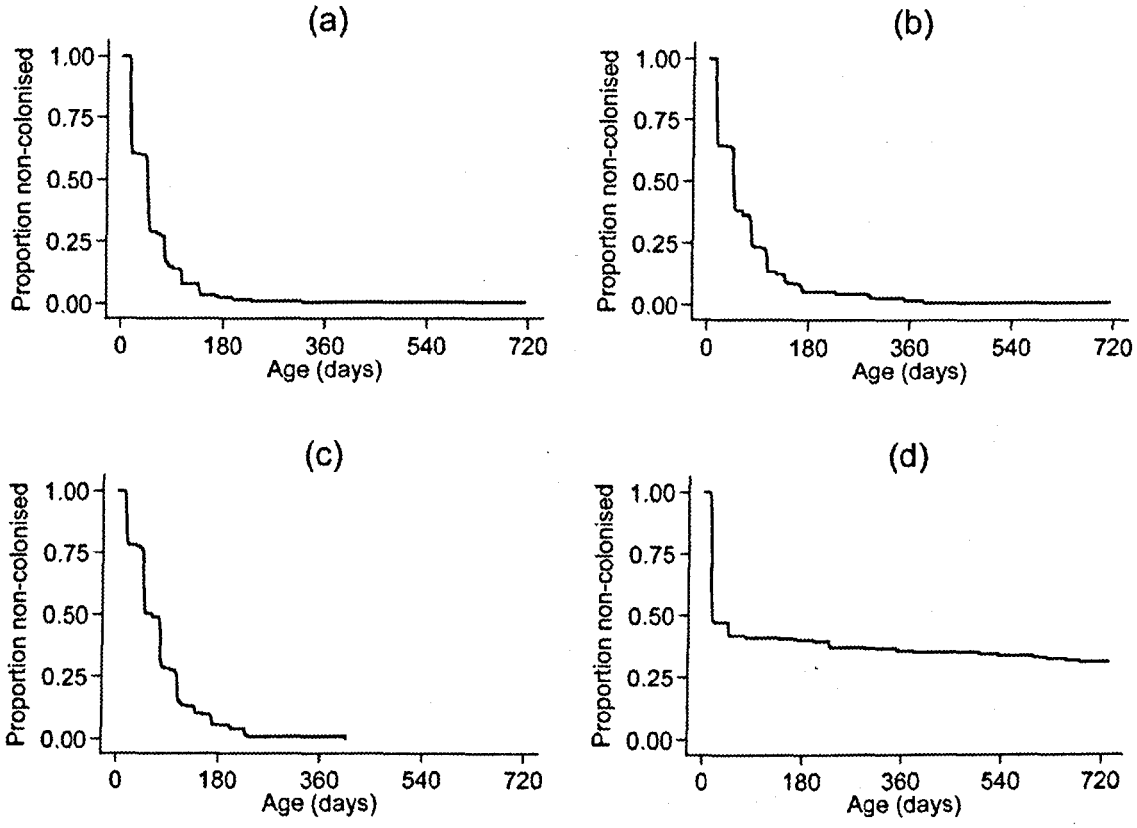
7.1.5.1. Colonisation in infants

7.1.5.1.1. Acquisition age

As previously described (section 3.1.5.1), infants acquired pneumococci at an early age (median 46 days, IQR 16 – 76) and all but one infant followed for more than one year carried pneumococci (180/181; 99.5%). *H. influenzae* acquisition characteristics were similar (median acquisition age 46 days, IQR 16 – 79) and all but one infant carried *H. influenzae* at least once (180/181; 99.5%). *Moraxella catarrhalis* was acquired slightly later (median 61 days, IQR 44 – 106) but all infants carried the organism at some stage. *S. aureus* was acquired very early (median 17 days (IQR 16 - ∞); however only 65.8% (119/181) of infants acquired the organism (Figure 33).

Figure 33. Time to first acquisition of nasopharyngeal colonisers in immunology infants

(a) S. pneumoniae, (b) *H. influenzae*, (c) *M. catarrhalis*, and (d) *S. aureus*.

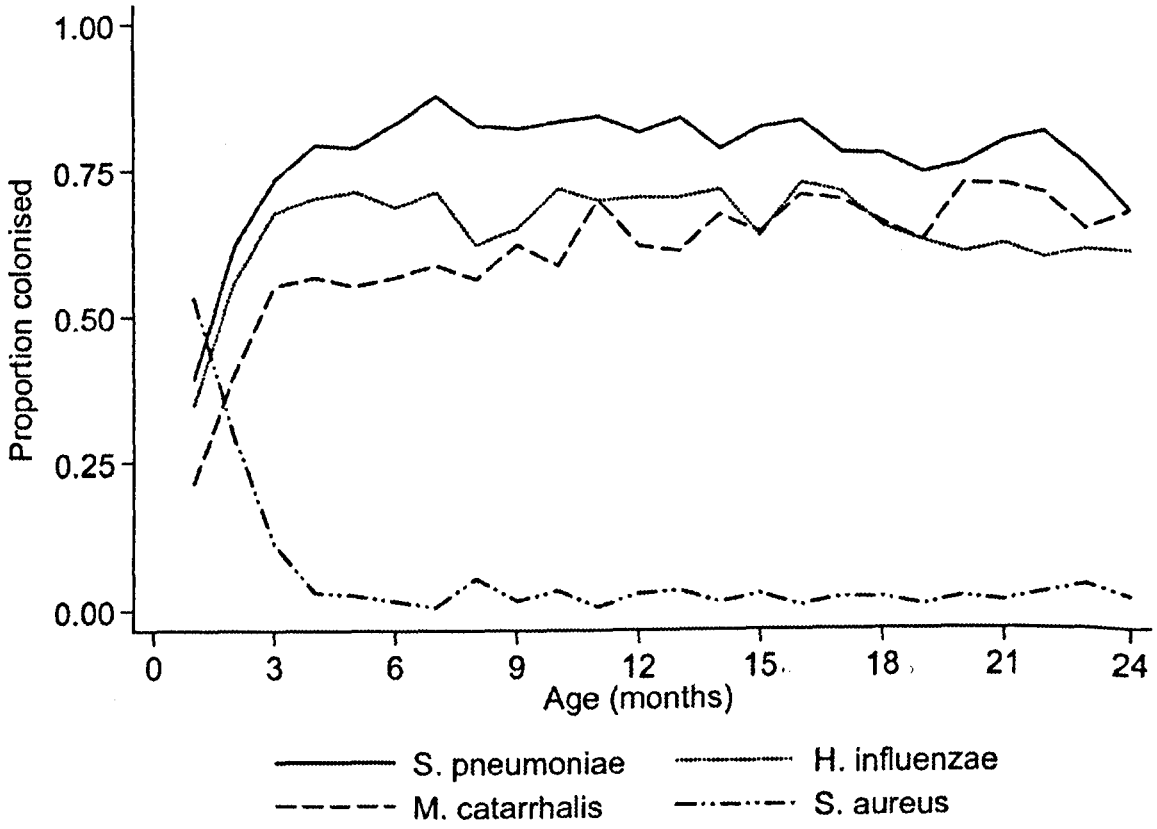


7.1.5.1.2. Monthly colonisation point prevalence

Streptococcus pneumoniae was cultured from 76.3% (3,192/4,182) of infant swabs. *H. influenzae* was cultured from 64.5% (2,696/4,182), *M. catarrhalis* from 59.4% (2,483/4,182), and *S. aureus* from 6.8% (286/4,182) swabs. Ninety five percent of *H. influenzae* isolates were non-typeable and 68.8% (99/144) of typeable isolates were type b. Monthly point prevalence of each organism is summarised in Figure 34. At the three month NPS visit, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* were detected in 73.2%, 67.5%, and 55.0% of swabs respectively. Monthly point prevalences for these organisms subsequently remained >50% for the remainder of the follow-up period. The opposite trend was observed for *S. aureus*: 53.0% of infants were colonised at the first

month visit and this proportion subsequently declined rapidly, with only 11.0% of three month swabs positive for the organism.

Figure 34. Point prevalence of nasopharyngeal colonisers in immunology infants

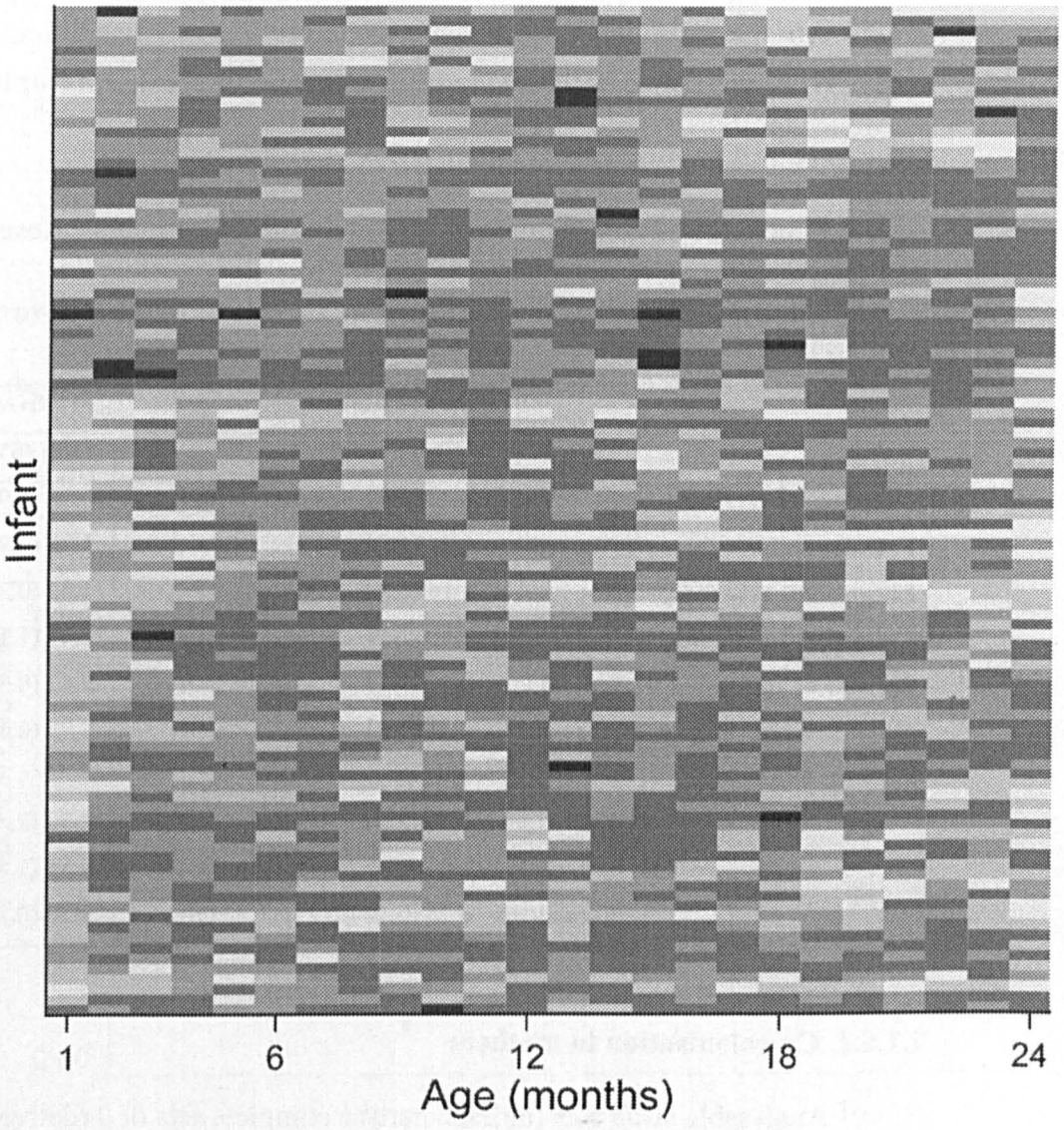


7.1.5.1.3. Associations between pneumococcal colonisation, other nasopharyngeal colonisers, and environment

Detection of pneumococci plus one (30.9%; 1,293/4,182) or two (37.2%; 1,555/4,182) additional target organisms was common, occurring in 68.1% of all infant NPS specimens. All four organisms were detected in 0.9% (38/4,182) swabs. *S. pneumoniae* was the only target organism detected in 9.6% (306/3,192) of swabs from which pneumococci were cultured. There was no obvious temporal association between the number of target organisms detected and infant age (Figure 35).

Figure 35. Heat map of nasopharyngeal colonisation by *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis,* and *Staphylococcus aureus*

The figure summarises 100 "immunology" follow-up group infants with complete sets of 24 monthly swabs. The number of species detected (from 0 - 4) is indicated by shading (light grey – black)). Each infant is represented by an individual row.



By univariate analysis, pneumococcal colonisation was positively associated with increasing age, female gender, co-habiting with other young children, detection of pneumococci in the preceding months swab, and colonisation by either *H. influenzae* or *M. catarrhalis*. Recent antimicrobial use and colonisation by *S. aureus* were negatively

associated with pneumococcal detection (Table 37). All factors were included in a multivariate logistic regression (GEE) model. Age, additional young children in the household, *H. influenzae* or *M. catarrhalis* colonisation remained positively associated with pneumococcal colonisation whereas antimicrobial use and *S. aureus* colonisation were negatively associated (Table 37).

Table 37. Associations between environmental factors, other nasopharyngeal colonisers and pneumococcal colonisation

Analyses included all 234 "immunology" follow-up group infants. Results are odds ratios with 95% CI for detection of *S. pneumoniae.*

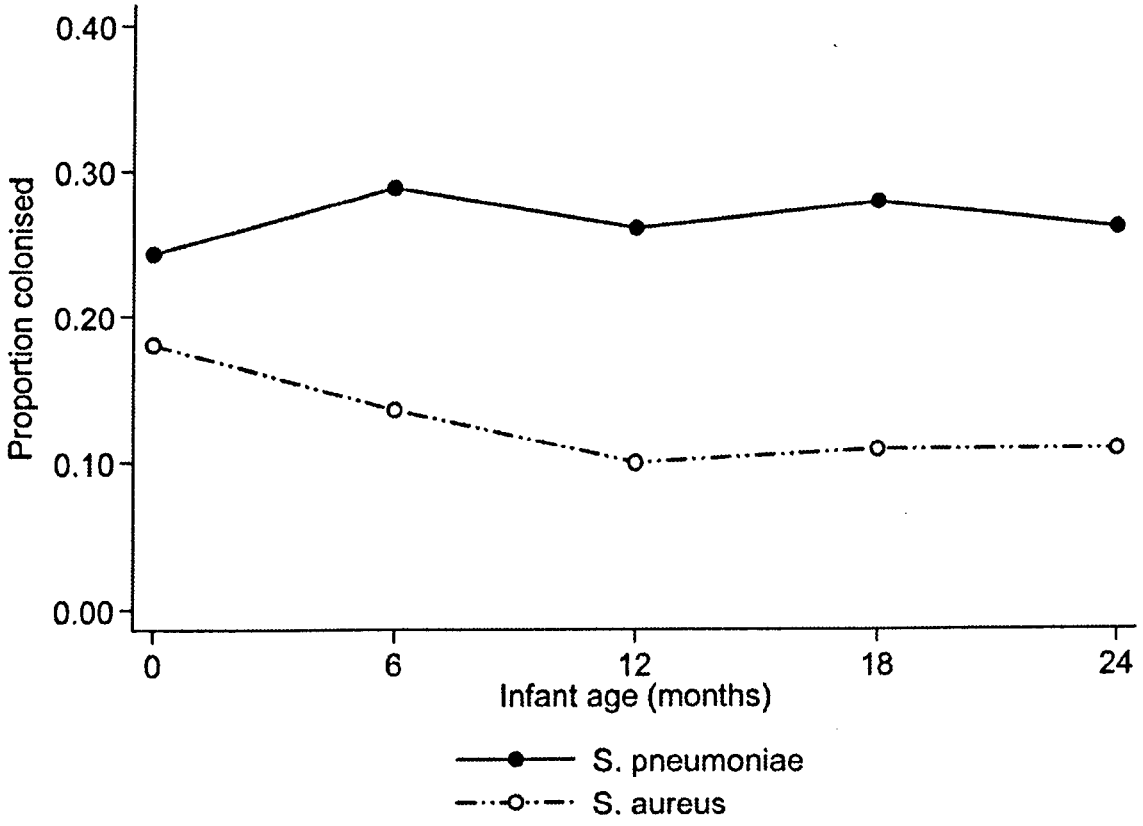
Factor	Univariate model		Multivariate model	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (/month increase)	1.03 (1.02 - 1.04)	< .001	0.98 (0.97 - 1.00)	.006
Female gender	1.34 (1.04 - 1.73)	.03	1.22 (0.99 - 1.51)	.07
Large household (>5 people)	1.16 (0.89 - 1.51)	.3	1.02 (0.82 - 1.27)	.8
Children <5y in house	1.70 (1.31 - 2.21)	< .001	1.41 (1.14 - 1.76)	.002
Episode of antibiotics within 30d	0.47 (0.39 - 0.58)	< .001	0.42 (0.34 - 0.54)	< .001
***S. pneumoniae* in preceding swab**	3.31 (2.83 - 3.86)	< .001	2.75 (2.31 - 3.28)	< .001
Colonisation by:				
H. influenzae	3.30 (2.84 - 3.85)	< .001	2.90 (2.46 - 3.43)	< .001
M. catarrhalis	2.41 (2.09 - 2.78)	< .001	2.14 (1.81 - 2.52)	< .001
S. aureus	0.18 (0.14 - 0.23)	< .001	0.30 (0.23 - 0.41)	< .001

7.1.5.2. Co-colonisation in mothers

Analysable swab sets (defined here as complete sets of 0 (delivery), 6, 12, 18, and 24 month NPS specimens) were available for 111 mothers. Pneumococci were detected in 26.7% (148/555) specimens, and PCV7 serotypes were found in 21 swabs (3.8%). *S. aureus* was detected in 12.6% (70/555) specimens and there was a trend towards a negative association with increasing infant age (OR 0.97, 95% CI 0.95 – 1.00, $P = .06$). In a crude analysis, there was a trend towards a negative association between overall pneumococcal

colonisation and *S. aureus* colonisation: pneumococci were detected in 17.1% (12/70) of cultures where *S. aureus* was also detected compared with 28.0% (136/485) of cultures where *S. aureus* was not detected ($P = .05$) (Figure 36). However, this association was lost when controlling for clustering using a GEE (OR 0.72, 95% CI 0.40 – 1.28, $P = .3$). Exclusion of NT pneumococcal colonisation from the analysis did not alter this finding (OR 0.92, 95% CI 0.47 – 1.79, $P = .8$). There was no specific association between colonisation by a PCV7 serotype and *S. aureus* colonisation (OR 0.83, 95% CI 0.20 – 3.48, $P = .8$).

Figure 36. Point prevalence of *Streptococcus pneumoniae* and *Staphylococcus aureus* nasopharyngeal colonisation in mothers



7.1.5.3. Colonisation during clinically diagnosed pneumonia episodes

Four hundred and sixty eight first episodes of pneumonia diagnosed by WHO clinical criteria were analysed. The median age at diagnosis was 205 days (IQR 118 – 329; range 10 – 726; Figure 37). Pneumonia was highly seasonal, with peaks occurring in the late rainy season (September to November) each year (Figure 38).

Pneumococci were detected in 73.3% (343/468) of the NPS specimens. At least one virus was detected in 66.2% (310/468) of the NPA specimens. RSV was the most commonly detected virus (203; 43.4% of NPA), followed by adenovirus (158; 33.8%), hMPV (39; 8.3%), and influenza viruses (30; 6.4%). Multiple virus detection was common, occurring in 23.7% (111/468) of NPAs. Virus detection was also highly seasonal, with a large peak of RSV detection coinciding with the peak of pneumonia incidence each year (Figure 38).

In agreement with the previous analyses of longitudinal data, preceding pneumococcal colonisation or colonisation by *H. influenzae* or *M. catarrhalis* were positively association with pneumococcal detection by univariate analysis. *S. aureus* colonisation was negatively associated with pneumococcal detection. By univariate analysis, RSV detection was negatively associated with pneumococcal detection and there was a trend towards a positive association between influenza virus detection and pneumococcal colonisation (Table 38 & Figure 39). In a multivariate logistic regression model, controlling for age, gender, season of diagnosis, recent antimicrobial use, and co-colonisation, RSV detection remained negatively associated with pneumococcal detection (OR 0.52, $P = .02$; Table 38).

Figure 37. Histogram of infant age at first pneumonia diagnosis

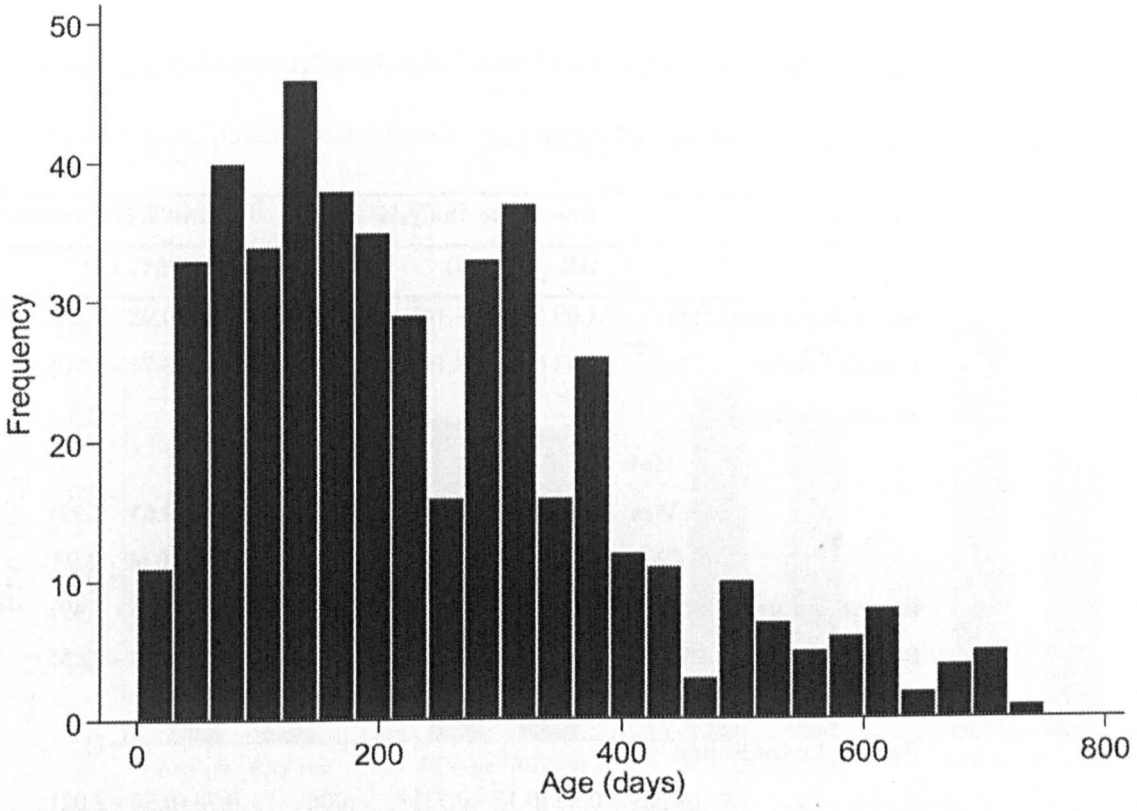


Figure 38. Seasonality of pneumonia diagnosis, pneumococcal colonisation, and virus detection

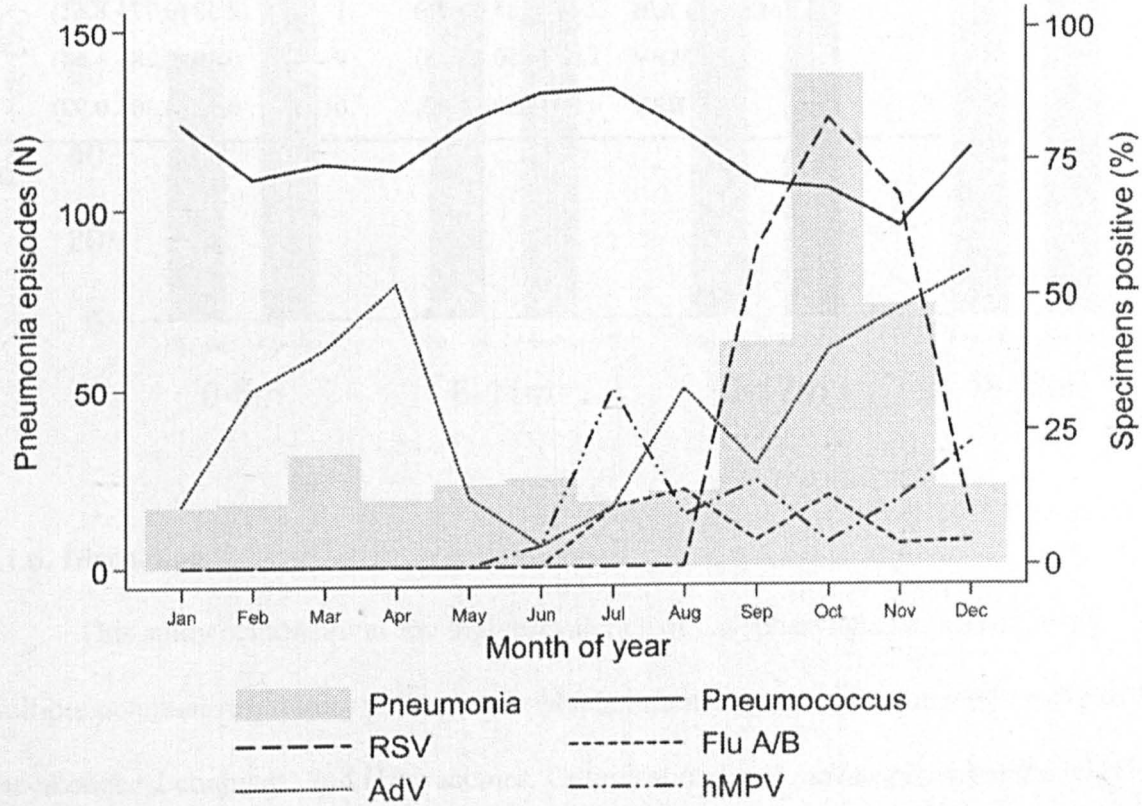


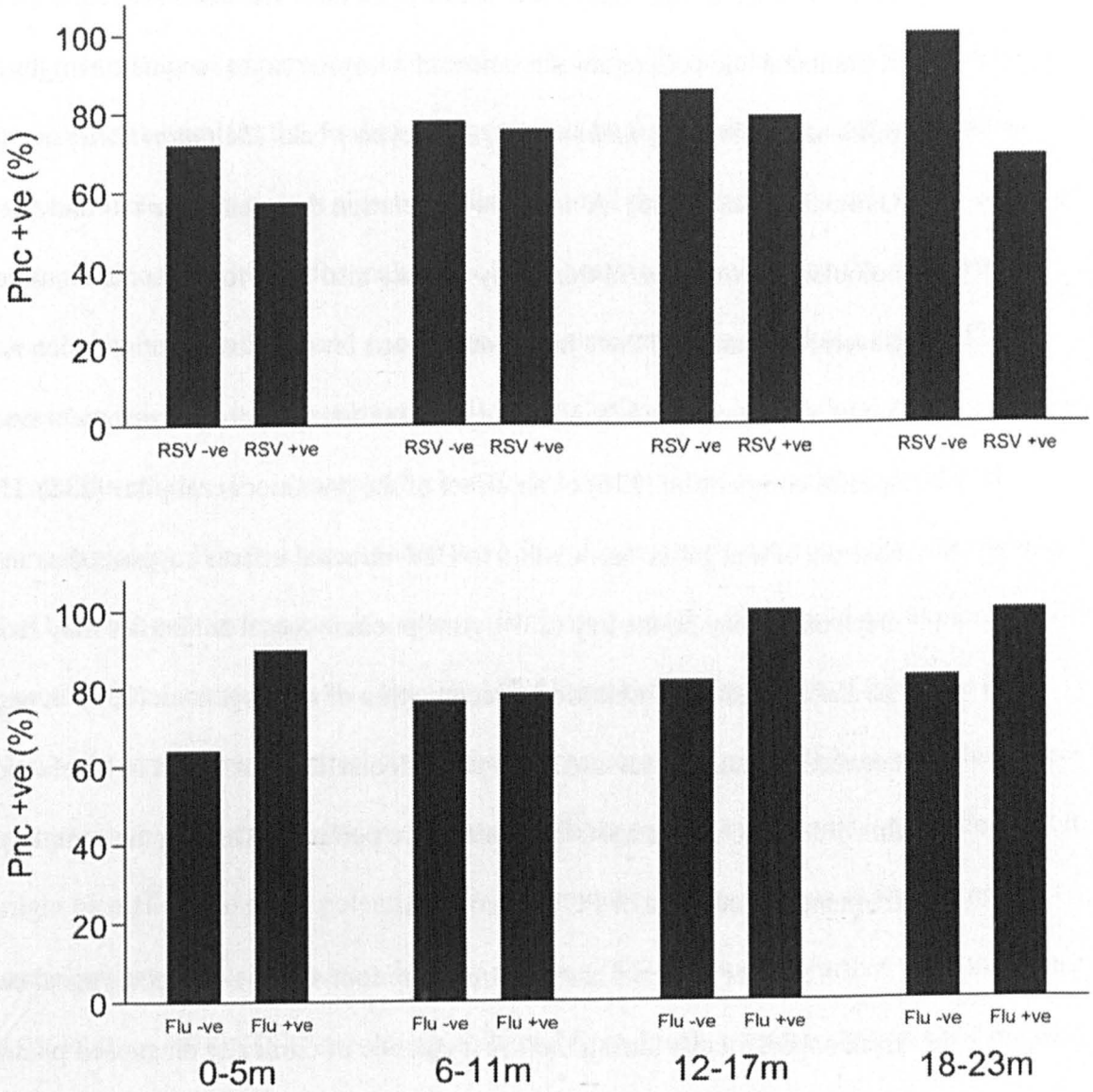
Table 38. Factors associated with pneumococcal colonisation detection at time of first WHO clinical pneumonia episode in 468 infants

The results are odd ratios (with 95% CIs) for detection of *S. pneumoniae.* All factors were included in the multivariate model.

Factor	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (/month increase)	1.09 (1.04 - 1.14)	< .001	1.03 (0.98 - 1.08)	.2
Female gender	1.43 (0.95 - 2.16)	.09	1.18 (0.74 - 1.90)	.5
Season at diagnosis				
Hot	-	-	-	-
Wet	0.86 (0.47 - 1.58)	.6	1.37 (0.63 - 2.99)	.4
Cool	0.67 (0.35 - 1.29)	.6	1.33 (0.60 - 2.97)	.5
Recent antibiotics (<30d)	0.55 (0.33 - 0.92)	.02	0.82 (0.45 - 1.49)	.5
Pneumococci identified in preceding swab (<45d)	8.41 (5.07 - 13.95)	< .001	7.20 (4.13 - 12.55)	< .001
Bacterial colonisation				
S. aureus	0.33 (0.15 - 0.73)	.006	0.78 (0.30 - 2.02)	.6
H. influenzae	2.18 (1.43 - 3.32)	< .001	2.06 (1.27 - 3.35)	.003
M. catarrhalis	1.94 (1.27 - 2.94)	.002	1.55 (0.84 - 2.55)	.09
Viral infection				
Adenovirus	1.11 (0.72 - 1.73)	.6	1.09 (0.66 - 1.81)	.7
Influenza A/B	2.48 (0.85 - 7.26)	.1	2.58 (0.77 - 8.61)	.1
hMPV	1.06 (0.50 - 2.25)	.9	0.68 (0.28 - 1.64)	.4
RSV	0.59 (0.39 - 0.90)	.01	0.52 (0.30 - 0.92)	.02

Figure 39. Pneumococcal colonisation status at pneumonia diagnosis by RSV and influenza virus PCR result and age group

The graph indicates the proportion of infants colonised with pneumococci at diagnosis of their first clinical pneumonia episode, stratified by virus detection (from the NPA collected at the same time point) and age group.



7.1.6. Discussion

This study demonstrates the high prevalence of nasopharyngeal colonisation by multiple potential respiratory pathogens in Maela infants, a population currently naive to pneumococcal conjugate and Hib vaccines. Colonisation by *S. aureus* preceded the other

target pathogens but was virtually absent after six months of age. Co-colonisation by *S. pneumoniae* and at least one of the other target organisms occurred frequently and was detected in around two-thirds of all infant NPS specimens. Detection of either *H. influenzae* or *M. catarrhalis* was significantly positively correlated with pneumococcal detection in infants. These results agree with previously published studies from similar populations, such as Papua New Guinea and rural Australia (48, 89, 105, 241). Using PCR, Kwambana and colleagues documented nasopharyngeal acquisition ages of three (*H. influenzae*), five (*S. pneumoniae*), and seven weeks (*M. catarrhalis*) in a cohort of Gambian infants (235). A negative association between *S. aureus* and *S. pneumoniae* colonisation was seen in this study and has also been found in other studies (233, 234). Several explanations have been put forward for this observation, which is most marked between *S. aureus* and PCV7 serotypes, including hydrogen peroxide-mediated inter-species competition (236) or an effect of the pneumococcal pilus (238). However, the absence of a negative association in HIV-infected infants suggests that an immunological mechanism may be the key (239). Anti-pneumococcal antibodies may cross-react with *S. aureus* and result in inhibition of acquisition of this organism (259). A negative association between pneumococcal and *S. aureus* colonisation was found in Maela mothers, although this did not achieve statistical significance perhaps reflecting the sample size and the low frequency of carriage of PCV7 serotypes.

Similar bacterial associations were found in the nasopharyngeal swabs collected from cohort infants during their first episode of clinically diagnosed pneumonia. As expected a positive association was seen between influenza virus infection and pneumococcal colonisation, although this failed to reach statistical significance, possibly as a result of the small number of proven influenza infections and the high prevalence of pneumococcal colonisation. Infection of the upper airways by influenza virus results in an increased load, greater adherence, and increase likelihood of transmissibility of pneumococci and this is mediated by a variety of incompletely understood mechanisms

(259, 260). RSV also increases pneumococcal adherence to the upper respiratory tract epithelium (261, 262). Pneumococcal conjugate vaccine reduced the number of virus-associated pneumonias in South African children, including a 22% fall in RSV-associated pneumonia (255). There is also a temporal association between RSV activity and invasive pneumococcal disease incidence (251). It was, therefore, unexpected to find a negative association between RSV detection and pneumococcal colonisation, especially given the high prevalence of pneumococcal colonisation the study infants. A plausible biological mechanism to explain this finding is elusive and further work is required to assess its veracity and importance. A significant limitation of the current study was the selection of only pneumonia episodes for inclusion of viral testing. It may be that the negative association between RSV and pneumococcal colonisation occurred as a result of this specimen selection bias. Jacoby and colleagues performed a longitudinal carriage study in Australian infants (0 – 2 years) which included bacterial and virus detection at each sampling point. Positive associations were found between rhinovirus and *S. pneumoniae* and between adenovirus and *M. catarrhalis*. Unfortunately, they could not explore RSV-pneumococcal interactions due to a very small number of detections of this virus (241). However, a recent case control study from Kenya rarely detected RSV in nasopharyngeal specimens from healthy children compared with those with pneumonia, suggesting that it might be difficult to study potential RSV-pneumococcal associations *in vivo* using nasopharyngeal specimens from healthy children (323). However, further work including RSV PCR on NPS-STGG specimens collected from Maela cohort infants at their monthly healthy follow-up visits would help to clarify the validity of this observation (324).

Decreasing costs and next-generation technology are resulting in sequenced-based analyses of the nasopharyngeal microbiome; indeed all NPS specimens from 20 Maela cohort infants are currently undergoing 16S sequencing at The Sanger Institute, UK (Dr Stephen Bentley). Studies to date have inevitably revealed far greater complexity to the colonising flora than previous culture-based studies, although none so far have had a

longitudinal component (245, 246). Interestingly, a Dutch study of 18-month old children found no clear association between viral nuclei acid detection and overall composition of the microbiome (245).

In conclusion, microbial interactions in the nasopharynx are complex and remain incompletely understood. The widespread introduction of pneumococcal conjugate vaccines, known to perturb the nasopharyngeal flora in culture-based studies, makes this an important area for on-going research. Improved detection of organisms by molecular approaches, coupled with collection of detailed immunologic and environmental data, will increase our understanding of the complex interactions between members of the nasopharyngeal microbiome.

8 Concluding remarks

The studies described in this thesis have defined the detailed characteristics of nasopharyngeal pneumococcal colonisation in mothers and infants living in an isolated SE Asian population.

8.1. Key findings

1. Pneumococcal colonisation occurred soon after birth and was persistent over the first two years of life. Infants were frequently co-colonised by *H. influenzae* and *M. catarrhalis*. A negative association was seen between colonisation by *S. aureus* and *S. pneumoniae.*
2. Risk factors for earlier pneumococcal acquisition in infants were environmental. Transplacentally acquired anti-pneumococcal antibodies did not significantly affect the timing of initial infant colonisation.
3. Previous exposure to a pneumococcal serotype did not generally reduce the risk of subsequent nasopharyngeal acquisition of the same serotype, although homologous serotype reacquisition was delayed and carriage duration shorter for the more immunogenic serotypes.
4. Pneumococcal acquisitions resulted in serotype-specific anti-capsular antibody responses which varied by serotype and generally increased with age. Infants developed serum IgG antibodies to a variety of pneumococcal proteins in response to colonisation. These responses were not strongly correlated with the overall number of pneumococcal acquisitions or colonisation duration. Antibody-mediated protection from nasopharyngeal colonisation was not observed.
5. Colonisation by antimicrobial resistant pneumococci was frequent and, in infants, associated with cohabitation with additional young children. Antimicrobial resistance was commoner in serotypes covered by the currently available conjugate vaccines.

6. Mothers acquired pneumococci less frequently, and carried them for shorter durations, than their infants. Transmission of pneumococci within the mother-infant pair declined with time.
7. The pneumococcal pilus did not have any demonstrable effect on transmissibility or colonisation duration of pneumococci.
8. WHO methodology significantly underestimates the prevalence of co-colonisation by multiple pneumococcal serotypes and latex sweep serotyping is a cost-effective improvement, although it is currently less effective at detection of serotypes present at low abundance.

8.2. General discussion

Whilst much is known about pneumococcal colonisation and subsequent infection, many uncertainties remain. Some of those uncertainties are slightly clearer as a result of this work.

Although perhaps not exciting science, the publication of robust serotype data from the pneumococci colonising a rural SE Asian population is important. Of course Maela is just one small rural community in a large, and heterogeneous, region. However, the region has been repeatedly noted to be poorly represented in the global *S. pneumoniae* dataset, despite having a high reported incidence of pneumonia, and no one study population will accurately reflect the entire region. Estimates of likely pneumococcal conjugate vaccine efficacy for SE Asia have either been inferred from other regions or based on very limited actual data. Whilst the gold-standard serotype data for vaccine planning purposes comes from examination of invasive disease isolates the characteristics of colonising pneumococci can be effectively used to inform vaccine strategy and future pneumococcal vaccine studies may use colonisation as the primary end-point of interest. Therefore the inclusion of the Maela serotype data in future meta-analyses for vaccine planning purposes will be useful.

Development and assessment of improved methodologies to increase the understanding of how pneumococcal serotypes interact in the nasopharynx is an important research area and highly relevant to vaccine development. Whilst the currently available PCVs have dramatically reduced the incidence of serious pneumococcal infection cause by vaccine serotypes, increases in non-vaccine serotype colonisation and infection have been noted. The development of novel pneumococcal protein-based vaccines with broader coverage may further reduce the prevalence of non-vaccine type pneumococci. However, on-going surveillance of pneumococcal colonisation to predict and subsequently monitor the effect of vaccine introduction will require methods that can more accurately capture the pneumococcal diversity in the nasopharynx, particularly the carriage of low abundance serotypes not covered by vaccine. Work contained in this thesis has demonstrated two methods, microarray-based or latex agglutination-based serotyping, that offer significant improvements in the detection of multiple serotype colonisation. Use of either of these methods in future colonisation studies would greatly enhance understanding of the complexity of pneumococcal interactions and how these are perturbed by immunisation. Choice of method would depend on the specific research question and study site/laboratory capability. The advantage of the latex sweep method is that it can be performed in laboratories with limited resources, assuming that appropriate quality control can be implemented.

The modifiers of pneumococcal colonisation remain incompletely understood. The studies described in this thesis have confirmed the bacterial associations reported in previous studies. Although both anti-capsular protein and anti-protein antibody responses to colonisation were examined in the Maela cohort, an unequivocally clearer signal was not forthcoming probably as a result of the near constant colonisation in many study individuals. Infants were able to respond to a variety of pneumococcal antigens, but an impact on serum antibody on colonisation could not be directly demonstrated. However, indirect evidence of immune-mediated effects on colonisation was noted. For certain

immunogenic serotypes, primary carriage episodes of the serotype were longer than when reacquisition of the homologous serotype occurred, suggesting improved immune mediate clearance. In Maela, a crowded refugee camp with many features similar to other developing world settings, household and environmental factors appeared to be the key determinants of pneumococcal colonisation dynamics in infancy.

8.3. Limitations

Inevitably, in retrospect, some aspects of the studies could have been improved by different study designs.

A shorter swabbing interval, at least for the first few months of life, may have improved the likelihood of detection of an effect of maternally-derived antibodies on timing of infant colonisation (71). It may also have resulted in more accurate determination of serotype-specific carriage durations, especially for the more rapidly cleared serotypes (285). However, estimates of acquisition rates and trends for carriage duration were not significantly different between the Maela study and those from studies of similar populations in The Gambia and Kenya (73, 74).

The mother was the only additional household member to be included in the pneumococcal colonisation work. This was a pragmatic choice, based on several factors including perceived likelihood of detection of transmission events compared with other household members. However, it limited the scope for analyses of pneumococcal transmission. It would have been preferable to include all household members in the carriage study (87).

The decision to limit viral detection work was a further limitation. Inclusion of viral testing of nasopharyngeal specimens from healthy follow-up visits would have allowed a more rigorous analysis of viral-bacterial interactions. Also, testing specimens for a broader panel of viruses would have improved this work. However, all specimens from the study were stored at -80 °C and preliminary work has determined that NPS-STGG specimens

may be an acceptable alternative to NPA-VTM specimens for detection of respiratory viruses (324). Therefore, further work on this important area remains possible.

The immunology work was restricted to the determination of serum IgG responses. Inclusion of analyses of mucosal and cellular immune responses would have strengthened this work (177, 178).

Despite the above, almost all of the aims and objectives stated in section 1.12 were addressed. The hypothesis that was not amenable to adequate testing was related to the relationships between colonisation and pneumococcal disease. As a result of the cohort size, there was only a single blood culture proven pneumococcal disease episode amongst study participants. Although it was of considerable interest to study relationships between pneumococcal colonisation and subsequent pneumonia episodes, the lack of definitively proven pneumococcal pneumonia, a factor common to almost all paediatric pneumonia studies, resulted in these analyses being beyond the abilities of this candidate. Approaches to mathematical modelling of the serotype colonisation data in relation to clinical and radiologic pneumonia episodes are under discussion, but ultimately this work may not be possible due the study sample size and uncertainty around the aetiologies of the pneumonia episodes. Restricting to x-ray confirmed pneumonia episodes would increase specificity for inferring pneumococcal infection, but since Hib vaccine was not available to study participants a certain degree of aetiological uncertainty would persist and also this approach would also erode the sample size.

8.4. On-going and future research to build on results from the thesis studies

The detection of multiple pneumococcal serotype colonisation is important, especially with the widespread introduction of PCVs. The latex sweep method is useful technique for detection of serotype co-colonisation and is well suited for use in resource-poor settings. However, further optimisation of the method is required to ensure that serotypes present at low relative abundance can be reliably detected. This work is planned.

Mathematical modelling of the cohort data is planned, and this will focus on determination of the characteristics and modifiers of pneumococcal transmission of within mother-infant pairs (Dr Ben Cooper, MORU). The frequent sampling and detailed meta-data will enhance these analyses. Future projects at SMRU are planned to study the impact of pneumococcal colonisation in pregnancy on infant outcomes, including infant colonisation and immune responses.

Culture-based studies of colonisation underestimate the complexity of the nasopharyngeal microbiome (245). Nasopharyngeal swabs from 20 Maela cohort infants are undergoing 16S sequencing at The Sanger Institute (Dr Stephen Bentley and Ms Zannah Salter). Previous studies have of the nasopharyngeal microbiome have been cross-sectional: this study will add an important longitudinal component.

Rapidly falling costs, and the development of next-generation sequencing technology such as Illumina HiSeq (Illumina Inc., San Diego CA, USA), has resulted in the reality of sequencing the genomes of entire populations of pneumococci. This approach has been successfully applied to a globally successful lineage of invasive pneumococci (33). More recently, the genomes of over three thousand GPS-mapped pneumococcal isolates collected during the Maela cohort study have been sequenced at The Sanger Institute (Pneumo3K, Dr Stephen Bentley and Ms Claire Chewapreecha). The aim of this study is to describe pneumococcal evolution and transmission in Maela at the genome level.

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11 Appendix 1: ARI study case record forms

11.1. Mother questionnaire

Date:
Study code: ARI -

Acute Respiratory Infection Study
Initial Questionnaire – Mother

Has Mother consented to take part in study and has she signed the consent form?

MLA code
Date of Birth (dd/mm/year) or age Ethnic Group
Address
Do you currently smoke (circle)? **YES/NO**
If yes what and how many do you smoke a day _____

Past medical history

1. Do you suffer from any medical conditions (circle)? **YES/NO**
   If yes what (circle) Asthma
   TB
   Heart problems
   Facial/ear malformations (circle)?
   Any other medical problems (circle)? **YES/NO**
   Details _____

2. Prescription of any antibiotics in the last 1 month (circle) **YES/NO**

Episode	1	2	3	4	5
What antibiotic?					
Started					
For how long (days)?					

3. Prescription of other medication in past 12 months (circle)? **YES/NO**

Episode	1	2	3	4	5
What medication?					
Started					
For how long (days)?					

Past Obstetric History

4. List previous pregnancies

Number	Year of Delivery	Preterm YES/NO	Sex of infant	Dead/Alive /Abortion

5. Have all previous pregnancies, deliveries and babies been normal (circle)? **YES/NO**
   If no please provide details:

History of Pregnancy

6. Date of last menstrual period ____/____/____ or unsure
7. EDD by USS ____/____/____ or not performed
8. Date USS first performed ____/____/____
9. Any abnormality found on USS (circle)? **YES/NO**
10. Any episodes of malaria during pregnancy (circle)? **YES/NO**
    Number of episodes _____
11. Any other illness during pregnancy (circle)? **YES/NO**
12. Any complications during this pregnancy (circle)? **YES/NO**

If answered yes to any of the above questions please provide details:

Family/Household

Accommodation

1. Number of rooms _____
2. Open fire in hut for cooking (circle)? **YES/NO**
3. What fuel is used on the fire? _____
4. Number of people living in the house in the last one month _____
5. Any family member's currently unwell (circle)? **YES/NO**

If yes details:

6. Details of household members:

Name	Relationship to infant	DOB or age (years) dd/mm/year	Work Y/N	Where work? Camp/outside/city
	Mother			
	Father			

11.2. Newborn examination

Date: __ __/__ __/__ __ **Newborn Examination**

Observations

1. What is the colour of the infant? (circle all that apply)
   Pale *Cyanosed* *Jaundiced* *Normal*

2. Is there a rash present? **YES/NO**
   Describe _____

3. Are there any birth marks present? **YES/NO**
   Describe _____

4. Describe the anterior fontanelle
   Raised *Normal* *Depressed*

5. Is there any bruising to the head or face? **YES/NO**
   Describe _____

6. Is the baby's face normal? **YES/NO**
   Describe _____

7. Are the palate and mouth normal? **YES/NO**
   Describe _____

8. Are the limbs, fingers and toes normal? **YES/NO**
   Describe _____

Cardiovascular Examination

9. Heart rate (per minute) _____ **If >160 or < 100 call the medic or doctor**

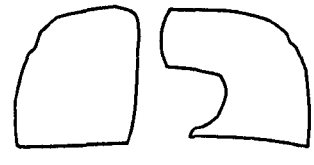
10. Describe the heart sounds
    Normal Murmur, describe _____

11. Are the femoral pulses present? **YES/NO**

Respiratory Examination

12. Respiratory rate (per minute) _____ **If >60 call the medic or doctor**

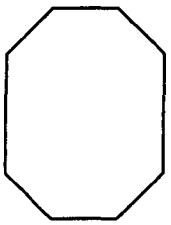
13. Listen to the chest is it clear? **YES/NO**
    If no describe the noise and location on the diagram



14. Are any of the following present? (circle all that apply)
    Chest indrawing *Nasal flaring* *Head bobbing* *None*

Abdominal Examination

15. On palpation of the abdomen, is there a mass / liver/ spleen palpable? **YES/NO**
    If yes please draw on the diagram



16. Is the genitalia normal (both testes descended in a boy)? **YES/NO**
    Describe _____

17. Does the infant have a normal anus? **YES/NO**
    Describe _____

Neurological examination

18. Does the infant have normal muscle tone? **YES/NO**
    If no describe _____

19. Is the infant moving all limbs normally? **YES/NO**
    If no describe _____

20. Does the infant have a normal red reflex? **YES/NO**

Is this a normal infant? YES/NO
If NO doctor or medic to record details (AND PHOTOGRAPH):

Outcome? (please circle)
Admitted IPD *Observation 24hours* *Home*

Is this infant in a study? **YES/NO**

If yes refer to study protocol
If no sign the form and add to ANC card

Infant next due to be seen _____

11.4. Monthly follow-up (0 – 12 month)

Study code: ARI- | **Address** _____

ANC code: MLA-

DOB (day/month/year): | **EGA:** _____ wks + _____ days | Where born?

Date of consultation:													
	Delivery	1	2	3	4	5	6	7	8	9	10	11	12
Anthropometry													
Weight (kg)													
Height (cm)													
Head Circum (cm)													
Arm Circum (cm)													
Feeding													
Feeding Code													
Add water in feeding y/n													
Diseases													
Anaemia y/n													
HCT %													
Malaria y/n													
LRTI y/n													
URTI y/n													
Otitis y/n													
Diarrhoea y/n													
Dysentery y/n													
Skin disease y/n													
Eye disease y/n													
Other problems y/n													
Medicine taken													
Antibiotics													
Vitamins													
Deworms													
Others													
Testing													
Nasopharyngeal swab													
Cord Blood													
Blood sample													

11.5. Monthly follow-up (13 – 24 month)

Study code: ARI- | **Address** _____

ANC code: MLA-

DOB (day/month/year): | **EGA:** _____ wks + _____ days

Date of consultation:												
	13	14	15	16	17	18	19	20	21	22	23	24
Anthropometry												
Weight (kg)												
Height (cm)												
Head Circum (cm)												
Arm Circum (cm)												
Feeding												
Feeding Code												
Add water in feeding y/n												
Diseases												
Anaemia y/n												
HCT %												
Malaria y/n												
LRTI y/n												
URTI y/n												
Otitis y/n												
Diarrhoea y/n												
Dysentery y/n												
Skin disease y/n												
Eye disease y/n												
Other problems y/n												
Medicine taken												
Antibiotics												
Vitamins												
Deworms												
Others												
Testing												
Nasopharyngeal swab												
Blood sample												

11.6. Infant illness

Date and time: Study code:

Date of Birth (dd/mm/year) Age at Examination (months)
Sex Weight (kg)

Has the child been assessed using the Emergency Protocol?

Has the child been in contact with dead or dying birds or with a household member with a severe respiratory disease? If yes, isolate the child and call the doctor.

History

1. Cough **YES/NO**
   If yes duration _____days

2. Fever **YES/NO**
   If yes duration _____days

3. Runny nose **YES/NO**
   If yes duration _____days

4. Noisy breathing **YES/NO**
   Describe _____

5. Earache **YES/NO**
   If yes duration _____days

6. Sore throat **YES/NO**
   If yes duration _____days

7. Vomiting **YES/NO**
   If yes duration _____days

8. Diarrhoea **YES/NO** If yes
   Duration _____days
   Frequency of stools _____/day
   Is there blood in the stool **YES/NO**

9. Rash **YES/NO**
   If yes duration _____days
   Describe the rash _____

10. Any problems with the baby's eyes? **YES/NO**
    Describe _____

11. When did the baby last pass urine _____(hours ago)

12. How is the baby being fed (circle)?
Breast feeding / Bottle Feeding / Mixed feeding / Other, give details _____

13. Does the baby suck well? **YES/NO**

Any other complaint by Mother describe here:

14.Has the baby taken any antibiotics or medicine since last seen? **YES/NO**

Episode	1	2	3	4	5
What medicine?					
Started					
For how long (days)?					

15.Does the baby have any medical problems such as heart problems, kidney problems or neurological problems? **YES/NO**
Details

16.Is the baby allergic to any medicines? **YES/NO**
Details

17.Are any family members unwell at the current time? **YES/NO**
Details

Examination
Observations
HR RR Temp O2 saturations Dextrose CRT

1. Is the infant (circle):
Alert / Sleepy / Irritable / Responding only to voice / Responding only to pain / Unresponsive

2. What is the colour of the infant (circle)?
Pale / Mottled / Cyanosed / Normal / Jaundiced

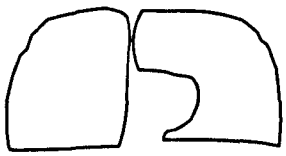
3. Are there any lymph nodes palpable? **YES/NO**
If yes where?_____

4. Is the infant dehydrated? **YES/NO**
If yes (circle) *Mild* *Moderate* *Severe*

5. Is the infant's cry normal? **YES/NO**
If no describe _____

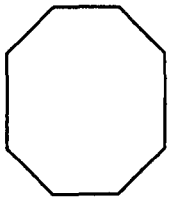
Cardiovascular Examination
1. Describe the heart sounds
Normal *Murmur*, describe _____

Respiratory Examination
1. Any of the following present (circle all that apply)?
Grunting *Stridor* *Chest indrawing*
Tracheal tug *Nasal flaring* *Head bobbing* *None*



2. Listen to the chest is it clear? **YES/NO**
If no, describe the noise and location on the diagram

Abdominal Examination
1. On palpation of the abdomen is there a mass / liver/ spleen palpable? **YES/NO**
If yes, please draw on the diagram



2. Are the genitalia normal? **YES/NO**
If no details

Neurological Examination

1. Does the infant have normal muscle tone? **YES/NO**
   Describe if no _____

2. Does the infant have normal posture? **YES/NO**
   Describe if no _____

3. Is the infant moving all limbs normally? **YES/NO**
   Describe if no _____

4. Are the infant's pupils equal and reactive? **YES/NO**
   Describe if no _____

5. Describe the anterior fontanelle (circle)
   Raised *Normal* *Depressed*

ENT Examination

1. Throat **Normal/Abnormal**
   If abnormal describe _____
2. Mouth **Normal/Abnormal**
   If abnormal describe _____
3. Ear **Normal/Abnormal if abnormal which one R/L/Both**
   If abnormal describe _____

Skin

1. Any, rash, pustules, cellulitis or skin abnormality? **YES/NO**
   Describe rash and location_____

Eyes

1. Any abnormality? **YES/NO**
   Describe if yes _____

Outcome *Home* *Home with follow up* *Admit IPD*

Diagnosis

Treatment (include length of treatment)

12 Appendix 2: ARI study laboratory standard operating procedures

12.1. Nasopharyngeal swab culture (SMRU SOP MBL-6-B)

12.1.1. Introduction

Nasopharyngeal swabs are obtained to determine the prevalence of carriage of respiratory pathogens (predominantly *Streptococcus pneumoniae* and *Haemophilus influenzae*).

12.1.2. Method

12.1.2.1. Specimen Collection and Transport

Nasopharyngeal samples are obtained with a deep nasopharyngeal swab. To obtain the specimen the patient's head should be tipped slightly backward and the swab passed directly backwards, parallel to the floor of the nasopharynx. The swab should pass without resistance until it reaches the posterior pharynx which is approximately one-half to two-thirds the distance from the nostril to the ear lobe. If resistance is encountered, the swab should be removed, and an attempt should be made to pass the swab through the other nostril. Once the swab is in place, rotate the swab 180 degrees or leave it in place for 5 s to saturate the tip before removing it slowly. The swab should be stored in cool box/refrigerator prior to and during transportation back to the laboratory.

12.1.2.2. Specimen Processing

Enter specimen details into the laboratory database/logbook.

Vortex collection vial (containing the swab and STGG medium) for 10-20 seconds.

Using a sterile 10µl loop, inoculate the following plates (in order):

1. Chocolate agar (+ bacitracin disc);
2. Columbia CNA agar with 5% sheep blood (BA-CNA).

Swabs from mothers should be inoculated onto BA-CNA only.

Incubate the Chocolate and BA-CNA plates overnight at 35-37°C in 3-10% CO_2.

Place the original specimen vial (with swab in situ) into the -80°C freezer.

NOTE: In most instances, swabs will be frozen -80°C at prior to culture. Before inoculating plates, ensure that the STGG is fully defrosted and mixed by vortex.

12.1.2.3. Follow-up

Read all plates.

Record the semi-quantitative growth of all colony types as follows:
1+ Growth in quadrant 1 but <10 colonies in quadrant 2;
2+ >10 colonies in quadrant 2 but <10 colonies in quadrant 3;
3+ >10 colonies in quadrant 3 but <10 colonies in quadrant 4;
4+ >10 colonies in quadrant 4.

Follow up and identify all potential pathogens* (as outlined below and in SOP MBL-4a/b-B):

- β-haemolytic streptococci;
- *Haemophilus influenzae*;
- *Moraxella catarrhalis*;
- *Staphylococcus aureus*;
- *Streptococcus pneumoniae.*

Only *Streptococcus pneumoniae* should be identified and followed-up from mother swabs.

Record the presence and quantity of all non-pathogenic colonisers (only perform minimal identification to confirm identity* – e.g. Gram stain, catalase, haemolysis type, and oxidase reactions).

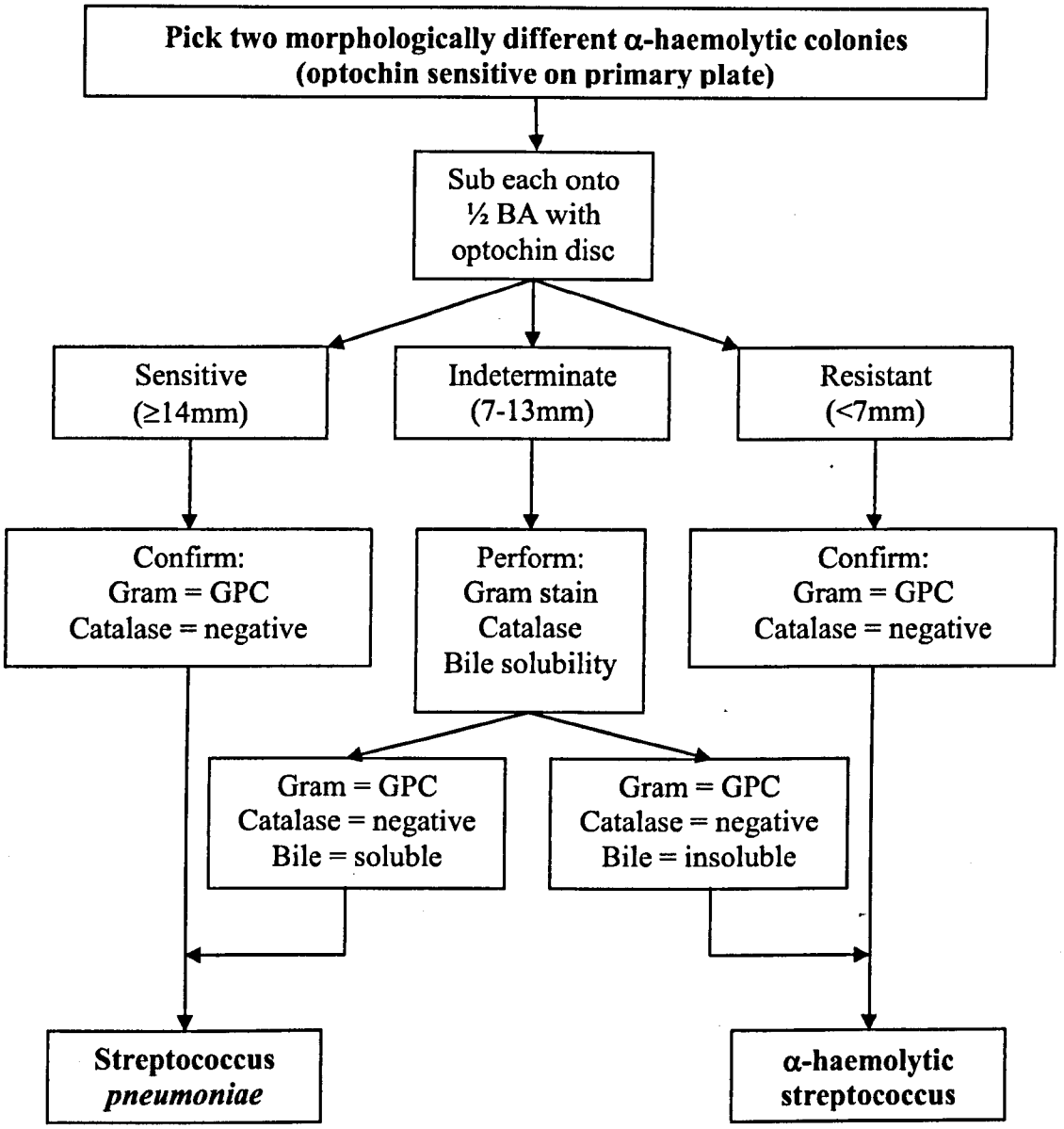
* Unless growth of a particular organism is heavy with isolated colonies, make a purity plate (¼ or ½ BA or Choc) before attempting identification tests.

Antimicrobial sensitivity testing is only to be done on isolates of *Streptococcus pneumoniae.* See SOP MBL-1-B.

Organism	**Identification methods**
Staphylococci	Catalase Coagulase / Staphaurex DNase agar
Streptococci	Catalase *Strep pneumoniae*: see below α-haem (opt R): no further ID required β-haem: group Enterococci: group sub onto bile-aesculin agar
GNR*	Record if LF/NLF on MacConkey Oxidase
Haemophili	Oxidase X and V factors (on nutrient agar) Test with anti-sera if *H. influenzae* β-lactamase test if *H. influenzae*
Moraxella	Oxidase Trybutyrin test β-lactamase test
Other organisms (e.g. yeasts)	Only if felt to be clinically significant

*If pure and heavy, consider full identification.

12.1.2.4. Follow-up of potential Streptococcus pneumoniae



All isolates of *Streptococcus pneumoniae* should be sub-cultured on to a plain blood agar (BA) plate and incubated overnight at 35-37°C in 3-10% CO_2.

From this sub-culture:

1. Perform antimicrobial sensitivity tests (see SOP MBL-1-B).
2. Serotype the isolate (see SOP MBL-20-B).
3. Save the organism (harvest growth from the whole plate with a sterile swab) in a labelled fresh vial of STGG medium and store in the -80°C freezer.

12.2. Pneumococcal serotyping (SMRU SOP MBL-20-B)

12.2.1. Introduction

There are over 90 serotypes of *Streptococcus pneumoniae.* Traditional serotyping of pneumococci is based on the capsular reaction (Quellung reaction): the result of an in situ immunoprecipitation between the pneumococcal capsular polysaccharide and its homologous antibody. A positive reaction is seen by use of a phase contrast microscope where the capsule becomes visible and the pneumococci agglutinates. This method is technically demanding, time consuming, and expensive. However, it remains the gold standard technique.

Alternative serotyping methods have been developed to overcome the problems of the Quellung reaction. An ideal method for large scale carriage studies, such as those in progress at SMRU, is one that is technically simple, rapid (to enable high throughput testing), and low cost. A latex agglutination method developed by Lafong and Crothers (*J. Clin. Pathol.* 1988;41;230-231) and modified by the MRC laboratories in The Gambia (Prof Richard Adegbola, personal communication) meets these criteria and is the primary method used at SMRU.

12.2.2. Method

12.2.2.1. Specimen Processing

Isolates are either processed in real-time (i.e. directly from the primary culture plate) or from sub-cultured frozen isolates (stored in STGG at -80°C).

12.2.2.2. A. Serotyping using the latex agglutination method

12.2.2.2.1. Day 1

Pick a single colony and sub-culture to a 5% sheep blood agar (BA) plate with an optochin disc positioned between the primary pool and 2nd streak.

Incubate the plate overnight at 35-37°C in 3-10% CO_2.

12.2.2.2.2. Day 2

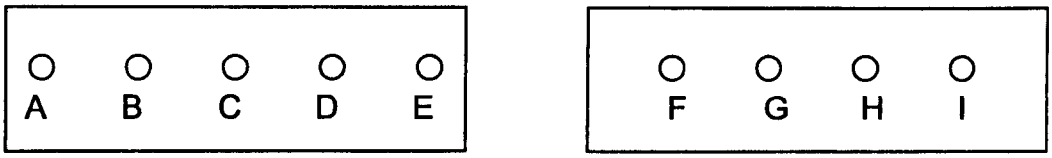
Check that the organism is pure and optochin sensitive (zone of $\geq$ 14mm). If mixed growth is suspected, re-culture a single colony on to a BA plate (and do not proceed with the mixed plate). If the organism is not optochin sensitive (i.e. zone of < 14mm), check a Gram stain, catalase reaction, and perform a bile solubility test (see Section C). If the organism is a GPC, catalase negative, and bile soluble proceed to step 4. If not, the organism is not a pneumococcus: re-check the original culture.

Remove the latex antisera from the reagent fridge and allow to warm up to room temperature. Mix thoroughly before use.

Using a sterile swab, make a light suspension (0.5 McFarland) of the organism in 2ml sterile saline.

Pipette 10µl of each pneumococcal pool latex antiserum (A-I) onto two labelled clean glass slides (see figure 1).

Figure 1



Add 10µl of the pneumococcal suspension to each drop of latex antiserum.

Mix and rotate for 2 minutes and observe for agglutination. A positive reaction for the particular pool is indicated by agglutination: proceed to step 10.

If no positive result is seen with the pool latex antisera, proceed to typing using the Quellung reaction, starting with Omniserum (see Section B). If a negative reaction is seen with Omniserum, check a Gram stain, catalase reaction, and perform a bile solubility test (see Section C).

Following the scheme outlined in Section E, repeat steps 6 - 8 using the appropriate group, type and factor specific latex antisera to determine the final serotype. Again, a positive reaction is indicated by agglutination.

Equivocal results must be confirmed by the Quellung reaction (see Section B).

Record the result on the serotyping results sheet.

12.2.2.3. B. Serotyping using the Quellung reaction

Prepare a heavy suspension of the test organism in a few drops of sterile saline.

Using sterile disposable loops, place 1µl each of methylene blue (0.3% solution) and the cell suspension onto a clean glass slide and mix. There should be a maximum of three drops per slide.

Add 1µl of the appropriate antiserum using a disposable loop. Include a negative control for each isolate (i.e. no antiserum added).

Cover with a cover slip and examine under an oil immersion objective lens. A positive reaction is seen as a change in the refractive pattern around the edge of the pneumococci. This gives the appearance of a thickening or swelling of the capsule. Comparison must be made with the negative control.

If there is a positive result with one of the antiserum pools, continue with full typing as outlined in Section E. If negative with antiserum pools A-I, try Omniserum.

If negative with Omniserum (and GPC, catalase negative, bile soluble) then record the isolate as non-typeable.

12.2.2.4. C. Bile solubility test

Two sterile polystyrene bijoux bottles are required for bile solubility testing of each suspect strain of *S. pneumoniae.*

Take a loop of the suspect strain from fresh growth on a blood agar plate and prepare a suspension (1-2 McFarland) in 0.5 ml of sterile saline.

Divide the suspension into two equal amounts (*i.e.*, 0.25 ml per bottle). Add 0.25 ml of saline to one tube and 0.25 ml of 10% sodium deoxycholate (10g Sodium deoxycholate in 100ml sterile distilled water) to the other.

Shake the tubes gently and incubate them at 35°– 37°C for 30 minutes.

Examine the tubes for lysis of cells in the tube containing the bile salts. A clearing of the tube, or a loss in turbidity, is a positive result.

12.2.2.5. D. Preparation of latex antisera

(Following the SOP of the MRC laboratories, Banjul, The Gambia: kindly provided by Prof Richard Adegbola)

12.2.2.5.1. Reagents

GBS - Glycine buffered saline (pH 8.2)

Glycine	1.5g
NaCl	11.7g
Distilled water	200ml

Adjust pH to 8.2 with 20%NaOH

GBS/BSA - Glycine albumin buffered saline

BSA	0.1g
GBS	100ml

Latex beads - 0.82μ diameter latex beads from Sigma (LB-8)

12.2.2.5.2. Preparation Procedure

Measure 1590μl of GBS into a labelled sterile bijou bottle.

Add 10μl of appropriate pneumococcal antiserum (from SSI).

Dilute the latex beads 1 in 8 in sterile saline in a universal container.

Add 1600μl of the dilute latex beads to the GBS-antiserum.

Incubate at 37°C in the waterbath for 2 hours shaking at 15 minutes intervals.

Add 3200μl of GBS/BSA.

Incubate overnight at 4°C.

Test with known bacterial suspension for quality assurance (e.g. ATCC 49619 – 19F).

Store in the reagent fridge.

Allow to warm up to ambient temperature before use.

12.2.2.6. E. Serotyping scheme

Pool	P	Q	R	S	T	Non-vaccine groups/types
A	1	18*	4	5	2	
B	19*	6*	3	8		
C	7*				20	24*, 31, 40
D			9*		11*	16*, 36, 37
E			12*	10*	33*	21, 39
F				17*	22*	27, 32*, 41*
G						29, 34, 35*, 42, 47*
H	14	23*		15*		13, 28*
I						25*, 38, 43, 44, 45, 46, 48

*Indicates that there are >1 types within the group

Type	Factor sera			
	6b	6c		
6A	+	-		
6B	-	+		
	7b	7c	7e	7f
7F	+	-	-	-
7A	+	+	-	-
7B	-	-	+	-
7C	-	-	-	+
	9b	9d	9e	9g
9A	-	+	-	-
9L	+	-	-	-
9N	+	-	+	-
9V	-	+	-	+
	10b	10d	10f	
10F	+	-	-	
10A	-	+	-	
10B	+	+	-	
10C	+	-	+	
	11b	11c	11f	11g
11F	+	-	-	+
11A	-	+	-	-
11B	+	-	+	+
11C	+	+	+	-
11D	+	+	-	-
	12b	12c	12e	
12F	+	-	-	
12A	-	+	-	
12B	+	+	+	
	15b	15c	15e	15h
15F	+	+	-	-
15A	-	+	-	-
15B	+	-	+	+
15C	-	-	+	-
	16b	16c		
16F	+	-		
16A	-	+		
	17b	17c		
17F	+	-		
17A	-	+		

Type	Factor sera				
	18c	18d	18e	18f	
18F	+	-	+	+	
18A	-	+	-	-	
18B	-	-	+	-	
18C	+	-	+	-	
	19b	19c	19f	7h	
19F	+	-	-	-	
19A	-	+	-	-	
19B	-	-	-	+	
19C	-	-	+	+	
	22b	22c			
22F	+	-			
22A	-	+			
	23b	23c	23d		
23F	+	-	-		
23A	-	+	-		
23B	-	-	+		
	24c	24d	24e		
24F	-	+	-		
24A	+	+	-		
24B	-	-	+		
	25b	25c			
25F	+	-			
25A	-	+			
	28b	28c			
28F	+	-			
28A	-	+			
	32a	32b			
32F	+	-			
32A	+	+			
	33b	33e	33f	6a	20b
33F	+	(+)	-	-	-
33A	+	(+)	-	-	+
33B	-	-	+	-	-
33C	-	+	(+)	-	-
33D	-	-	+	+	-
	35a	35b	35c	29b	42a
35F	+	+	-	-	-
35A	+	-	+	-	-
35B	+	-	+	+	-
35C	+	-	+	-	+
	41a	41b			
41F	+	+			
41A	+	-			
	47a	43b			
47F	+	-			
47A	+	+			

12.3. Detection of multiple pneumococcal serotype colonisation by latex agglutination

(SMRU SOP MBL-24-B)

12.3.1. Introduction

There are over 90 serotypes of *Streptococcus pneumoniae.*

Multiple serotypes may be carried concurrently in the nasopharynx, but detection of these by conventional culture and Quellung serotyping is costly, time-consuming and inefficient.

A latex agglutination serotyping method has been used to detect multiple pneumococcal carriage by the MRC laboratories in The Gambia. This method is described in the current SOP (see also SOP MBL-20-B – Pneumococcal serotyping).

12.3.2. Method

12.3.2.1. Day 1

NOTE: In most instances, nasopharyngeal swabs will be frozen at -80°C at prior to culture. Before inoculating plates, ensure that the STGG is fully defrosted and mixed by vortex.

Vortex the cryotube (containing the swab and STGG medium) for 10-20 seconds.

Using a sterile 10µl loop, inoculate a Columbia CNA agar with 5% sheep blood (BA-CNA) plate.

Incubate the plate overnight at 35-37°C in 3-10% CO_2.

Place the original specimen vial (with swab in situ) back into the -80°C freezer.

12.3.2.2. Day 2

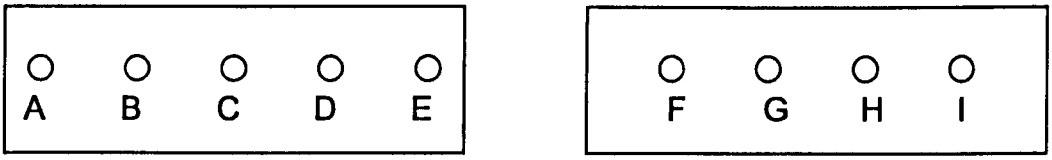
Check growth on the plate:
If ≥1+ growth of α-haemolytic colonies, proceed to step 2.
If <1+ growth of α-haemolytic colonies, re-subculture 50µl STGG on to a BA-CNA plate.

Remove the latex antisera from the reagent fridge and allow to warm up to room temperature. Mix thoroughly before use.

Using a sterile swab, make suspend all of the colonies from the plate in 0.5 - 2ml sterile saline (to make at least a density of 0.5 McFarland).

Pipette 10µl of each pneumococcal pool latex antiserum (A-I) onto two labelled clean glass slides (see figure 1).

Figure 1



Add 10µl of the organism suspension to each drop of latex antiserum.

Mix and rotate for 2 minutes and observe for agglutination. A positive reaction for a particular pool is indicated by agglutination.

Following the scheme outlined in SOP MBL-20-B, repeat steps 6 - 8 using the appropriate group, type and factor specific latex antisera to determine the final serotype(s). Again, a positive reaction is indicated by agglutination.

Record the results on the serotyping results sheet.

A NPS may contain >1 pneumococcal serotype: follow-up all positive reactions according to the typing scheme (See SOP MBL-20-B).
It is possible to determine the presence of non-typeable pneumococci in a culture: a weak reaction with Pool B and Group 19 latexes, but no reaction with the Group 19 factor latexes, is observed.

12.4. Respiratory virus PCR (SMRU SOP MBL-34-P)

12.4.1. Introduction

Several important respiratory viruses are sought using real-time RT-PCR (rRT-PCR, Taqman):

- Adenovirus (Adeno)
- Influenza A (FluA), Swine influenza A [2009 H1N1] (SwFluA), influenza B (FluB), plus sub-typing assays for influenza A (H1, H3, and SwH1)
- Human metapneumovirus (hMPV)
- Respiratory syncytial virus (RSV)

US-CDC assays are used to detect Adeno, FluA, SwFluA, FluB, and RSV. A previously published assay is used for hMPV (Maertzdorf J et al., J Clin Mic 2004). Detection of the human RNaseP gene is used as an internal/specimen control (Emery SL et al., Emerg Inf Dis 2004). Periodically, additional respiratory viruses may be sought (e.g. rhinoviruses and parainfluenza 1-4) using commercial PCR kits.

12.4.2. Important points

12.4.2.1. Specimens

A nasopharyngeal aspirate (NPA) is the specimen of choice, although combined nose and throat swabs may be used for detection of influenza viruses). CDC protocols state the following:

12.4.2.1.1. Acceptable specimens:

- Respiratory specimens including: bronchoalveolar lavage, tracheal aspirates, sputum, nasopharyngeal or oropharyngeal aspirates or washes, and nasopharyngeal or oropharyngeal swabs.
- Swab specimens should be collected only on swabs with a Dacron tip and an aluminium or plastic shaft. Swabs with calcium alginate or cotton tips and wooden shafts are unacceptable.

12.4.2.1.2. Specimen rejection criteria:

- Specimens not kept at 2-4°C (for up to 4 days) or frozen at -70°C or below.
- Incomplete specimen labelling/documentation.
- Insufficient specimen volume.

12.4.2.2. PCR technique

Meticulous technique is required for consistent PCR results – refer to the SMRU "Working in the PCR laboratory SOP".

12.4.2.3. Reagents required

- Invitrogen SuperScript III Platinum One-Step Quantitative RT-PCR Kit.
- Sterile distilled water (RNase and DNase free).
- Forward and reverse primers (See SOP MBL-32-P – Microbiology PCR primers and probes).
- Dual-labelled probes (See SOP MBL-32-P – Microbiology PCR primers and probes).
- Positive control virus RNAs (Appendix B).
- Clinical specimens.

12.4.2.4. Equipment required

- Pipettes and sterile barrier pipette tips
  - 0.1ml or 0.2ml PCR reaction tubes (0.1ml: Corbett, cat. no. 3001-002; 0.2ml: Axygen)
- Cold racks for 1.5 microcentrifuge tubes and 0.2ml PCR reaction tubes
- Sterile 1.5 ml microcentrifuge tubes
- Powder-free gloves
- Microcentrifuge
- Vortex mixer
- Real-time PCR detection system (Corbett Rotorgene 6000)

12.4.3. Method

12.4.3.1. Equipment preparation

- Work surface, pipettes and centrifuges should be cleaned and decontaminated with 10% bleach followed by 70% alcohol to minimize risk of contamination: prepare fresh bleach solution each day.
- Ensure cold racks are frozen.

12.4.3.2. Reagent preparation

- Remove the clinical specimen extracts from the -80°C freezer and put in the class II cabinet in the DNA/RNA extraction laboratory to defrost.

The following steps are carried out in cabinet, in the reagent preparation laboratory:

- Keep all reagents on cold rack during assay set up.

(a) Primers and probes

- Thaw frozen aliquots of primer and probes (thawed aliquots of primers and probes may be stored in the dark up to 3 months at 2-8°C; label with an expiry date).
- Vortex all primers and probes.
- Briefly centrifuge all primers and probes and then place in cold rack.

(b) Real-time RT-PCR reagents

- Place Master Mix and enzyme in cold rack.
- Thaw the 2X Reaction Mix vial.
- Mix the 2X Reaction Mix by vortexing for 15 sec.

- Briefly centrifuge 2x Reaction Mix and enzyme (Taq mix) then place in cold rack.

12.4.3.3. Setting up the rRT-PCR reaction

12.4.3.3.1. Controls included in each rRT-PCR run

- No Template Control (NTC):
  - Validates reagent integrity (negative).
- Viral Template Control (VTC):
  - Validates reagent integrity (positive).
- Mock Extraction Control (MOCK):
  - Validates the extraction procedure and reagent integrity (negative).

12.4.3.3.2. Assay set up

- The following assays can be combined on the same rRT-PCR run (same reaction conditions):
  - RNP + FluA + SwFluA + FluB + FluA typing (H1 + H3 + SwH1)
  - Adeno +RSV
- hMPV is performed on a separate rRT-PCR run (different reaction conditions).
- Maximum number of specimens on a PCR run:
  - Single virus = 69 (+3 controls);
  - Rapid influenza detection [RNP + FluA + SwFluA + FluB] = 15 (+12 controls).
- Reaction assay mixtures are made as a cocktail ("the master mix") and dispensed into the reaction tubes (0.1ml or 0.2 ml depending on the number of specimens).
- Extracted nucleic acid, viral template controls, or water is then added to the appropriate test reactions and controls.

In the office area:

- Calculate the amounts of reagent required (see Appendix A) for the master mix.
- The electronic master mix worksheet is located on the SMRU server.

The following steps are carried out in the cabinet, in the reagent preparation laboratory:

- Label the reactions tubes on the lid according to the scheme from the worksheet and place in a cold rack.
- Make up the master mix:
  - Mix reagents by pipetting up and down.
- Add 20 µl of each master mix into each tube.
- Before moving the plate to the DNA/RNA extraction laboratory, set up the NTC reactions in the reagent preparation laboratory:
  - Pipette 5 µl of nuclease free water into the NTC tubes.
  - Cap NTC tubes.

Now move to the DNA/RNA extraction laboratory:

- Ensure that the clinical specimen extracts are fully defrosted (stored at -80°C following extraction).

- Make a 1:10 dilution of extracted nucleic acid (this step improves performance in the RNaseP assay – removes false negatives; unpublished data): add 10 µl of extract to 90 µl nuclease free water.
- Vortex for a few seconds the tubes containing the clinical specimen extracts.
- Briefly spin the extracts in the microcentrifuge.
- Pipette 5 µl of the extract into all the tubes labelled for that specimen (e.g., specimen "S1", Adeno + RSV). Cap the tubes immediately after adding the extracted RNA.
- Repeat the above steps for the remaining specimen extracts.
- Add 5 µl of mock extracted sample to the MOCK tubes. Cap the MOCK tubes.
- Finally, pipette 5 µl of positive control into all VTC tubes. Cap the VTC tubes.

Now move to the PCR amplification laboratory:

- Switch on the Rotorgene 6000 machine and laptop.
- Load the specimens in the order described in the worksheet (NTC, clinical specimens, MOCK, VTC).
- Select the correct rRT-PCR run file.
- Start the rRT-PCR run.
- Copy and paste the run details (assay set up) into the Rotorgene software from the worksheet.
- At the end of the run, analyse the results and print (and save the report).
- Remove the tubes from the machine without opening and put in a sealed plastic bag for disposal (red bag).
- When all assays are completed for a specimen, enter the data into the Microbiology laboratory database.

12.4.4. Interpretation and limitations (from CDC protocols)

12.4.4.1. RNP PCR (Internal/specimen control)

All clinical samples should be exhibit RNP reaction curves that cross the threshold line at or before **35 C_T**, indicating the presence of the human RNase P gene.

Failure to detect RNase P in any of the clinical samples may indicate:

- Improper extraction of nucleic acid from clinical materials resulting in loss of RNA or carry-over of RT-PCR inhibitors from clinical specimens.
- Absence of sufficient human cells in sample to enable detection.
- Improper assay set up and execution.
- Reagent or equipment malfunction.

If a specimen is RNP negative, re-extract and repeat the PCR (on the next run).
If all of the specimens are RNP negative (including the VTC), suspect PCR failure and repeat the assay.

12.4.4.2. Adeno, FluA, SwFluA, FluB, hMPV, and RSV rRT-PCR

A sample is considered positive when a curve crosses the threshold before **40 C_T**.

This is only true if all quality controls worked properly (i.e. the NTC and mock must be negative, and the VTC positive [within 2S.D. of the mean using the process control chart]). If any MOCK or NTC is positive, or a VTC is negative, then the run is invalid since either contamination or reagent failure has occurred. Repeat the PCR with fresh reagents.

Additionally, samples with virus PCR C_T values between 35 and 40 are considered low positive: these results must be confirmed by repeating the PCR on the sample in duplicate on the next run (do not repeat all PCR, just the one with the low positive result).

12.4.4.3. Limitations

The presence of excess DNA/RNA template in the specimen may result in false negative results. If a negative result is obtained and high levels of nucleic acid are suspected, the extracted sample may be tested at 2 or more dilutions (e.g. 1:10 and 1:100) to verify the result.

12.4.5. Appendix A: Specific rRT-PCR assay details

12.4.5.1. Master mixes (25µl reaction volume)

- Label one 1.5 ml microcentrifuge tube for each primer/probe set.
- Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction cocktail for the MOCK, NTC and VTC reactions and for pipetting error:
    - If the number of samples (n) including all controls = 1 to 14, then N = n + 1
    - If the number of samples (n) including all controls > 15, then N = n + 2

12.4.5.1.1. FluA, SwFluA, FluB, FluA sub-typing, and RNP (CDC)

Reagent	Per tube (µl)
Taq Mix	N x 0.50
2X Reaction Mix	N x 12.50
Forward primer	N x 0.50
Reverse primer	N x 0.50
Probe	N x 0.50
Nuclease-free water	N x 5.50
Total Volume	**N x 20.00**

12.4.5.1.2. Adeno and RSV (CDC) & hMPV (Maertzdorf)

Reagent	Per tube (µl)
Taq Mix	N x 0.50
2X Reaction Mix	N x 12.50
Forward primer	N x 0.25
Reverse primer	N x 0.25
Probe	N x 0.25
Nuclease-free water	N x 6.25
Total Volume	**N x 20.00**

12.4.5.2. Reaction conditions

12.4.5.2.1. FluA, SwFluA, FluB, FluA sub-typing, and RNP (CDC)

Reverse Transcription	50°C for 30 min
Taq inhibitor inactivation	95°C for 2 min
PCR amplification (45 cycles)	95°C for 15 sec 55°C for 30 sec

12.4.5.2.2. Adeno and RSV (CDC)

Reverse Transcription	50°C for 30 min
Taq inhibitor inactivation	95°C for 2 min
PCR amplification (45 cycles)	95°C for 15 sec 55°C for 1 min

12.4.5.2.3. hMPV (Maertzdorf)

Reverse Transcription	50°C for 30 min
Taq inhibitor inactivation	95°C for 5 min
PCR amplification (45 cycles)	95°C for 30 sec 58°C for 1 min

12.4.6. Appendix B: Positive controls (VTC)

Each rRT-PCR run must include a positive control. Either virus controls from CDC or plasmid controls (SMRU) are used.

The C_T value of the positive control must be established prior to introduction and should be around 30. Positive control material should be aliquoted into single use tubes and stored at -80°C prior to use.

The batch number and preparation date of the positive control is recorded on every rRT-PCR worksheet.

After each rRT-PCR run, the C_T value of the positive control is entered into the Microbiology laboratory database and plotted on the appropriate process control chart. If the C_T value of the positive control is greater than 2S.D. from the mean value for the current batch, then corrective action must be taken:

- Repeat the rRT-PCR run with freshly made primers/probe.
- Consider the need for a new batch of positive control.
- Consider the need for a new *Taq* kit.

12.5. Primer and probe sequences (extracted from SMRU SOP MBL-32-P)

12.5.1. Introduction

This SOP describes the sequences, stock and working concentrations and general handling of PCR primers and probes for all SMRU microbiology laboratory PCRs.

12.5.2. Important points

Never use primers/probes that have expired.

12.5.2.1. Primer/probe stocks

- On receipt of a new batch of primers/probe, dilute as directed by the manufacturer (usually to a concentration of 100µM).
- Aliquot the stock into single use tubes (each tube containing sufficient volume to make a 100µl aliquot of working concentration primer/probe):
    - Probes must be protected from light: either cover the tube with foil or use a brown opaque tube.
    - Label each tube with the details of the primer/probe (name and concentration) and the expiry date (always one year after the preparation date).
    - Store stock concentration primers and probes in the -20°C freezer in the reagent preparation room. Do not re-freeze after thawing. Primers and probes may be stored this way for a maximum of one year.

12.5.2.2. Working concentration primers/probes

- For each PCR, only a single 100µl aliquot of working concentration primer/probe should be made at one time:
    - Probes must be protected from light: either cover the tube with foil or use a brown opaque tube.
    - Label each tube with the details of the primer/probe (name and concentration) and the expiry date (always three months after the preparation date).
    - Store working concentration primers and probes in the fridge in the reagent preparation room. Do not re-freeze after thawing. Primers and probes may be stored this way for a maximum of three months.
- New aliquots of primer/probe should be tested in parallel with the old batch to ensure activity prior to general use.

12.5.3. Primer and probe details

Assay Name (Ref)	Primers/Probe	Sequence (5'>3')	[Stock]	[Working]
Influenza A (CDC)	InfA_F	CDC – SEQUENCE NOT RELEASED	100 µM	40µM
	InfA_R	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	InfA_P	CDC – SEQUENCE NOT RELEASED	Dry	10 µM
Influenza A H1 (CDC)	InfA H1_F	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	InfA H1_R	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	InfA H1_P[1]	CDC – SEQUENCE NOT RELEASED	Dry	10 µM
Influenza A H3 (CDC)	InfA H3_F	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	InfA H3_R	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	InfA H3_P	CDC – SEQUENCE NOT RELEASED	Dry	10 µM
Swine Influenza A (CDC)	SW InfA_F	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	SW InfA_R	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	SW InfA_P[1]	CDC – SEQUENCE NOT RELEASED	Dry	10 µM
Swine Influenza A (CDC) H1	SW H1_F	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	SW H1_R	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	SW H1_P[1]	CDC – SEQUENCE NOT RELEASED	Dry	10 µM
Rnase P (Emery)	RnaseP_F	AGA TTT GGA CCT GCG AGC G	100 µM	40 µM
	RnaseP_R	GAG CGG CTG TCT CCA CAA GT	100 µM	40 µM
	RnaseP_P	TTC TGA CCT GAA GGC TCT GCG CG	Dry	10 µM
Influenza B (CDC)	InfB_F	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	InfB_R	CDC – SEQUENCE NOT RELEASED	100 µM	40 µM
	InfB_P	CDC – SEQUENCE NOT RELEASED	Dry	10 µM
RSV (CDC)	RSV_F	GGC AAA TAT GGA AAC ATA CGT GAA	100 µM	50 µM
	RSV_R	TCT TTT TCT AGG ACA TTG TAY TGA ACA G	100 µM	25 µM
	RSV_P	CTG TGT ATG TGG AGC CTT CGT GAA GCT	Dry	5 µM
hMPV (Maertzdorf)	NL_N_F	CAT ATA AGC ATG CTA TAT TAA AAG AGT CTC	100 µM	50 µM
	NL_N_R	CCT ATT TCT GCA GCA TAT TTG TAA TCA G	100 µM	25 µM
	NL_N_P	TGY AAT GAT GAG GGT GTC ACT GCG GTT G	Dry	10/50 µM*
Adenovirus (CDC)	Adeno_F	GCC CCA GTG GTC TTA CAT GCA CAT C	100 µM	50 µM
	Adeno_R	GCC ACG GTG GGG TTT CTA AAC TT	100 µM	50 µM
	Adeno_P	TG CAC CAG ACC CGG GCT CAG GTA CTC CGA	Dry	10 µM
Pneumo lytA (Carvalho)	lytA_CDC_F	ACG CAA TCT AGC AGA TGA AGC A	100 µM	10 µM
	lytA_CDC_R	TCG TGC GTT TTA ATT CCA GCT	100 µM	10 µM
	lytA_CDC_P	GCC GAA AAC GCT TGA TAC AGG GAG	Dry	10 µM
Pneumo 6A/C (UAB)	6A/C-fwd (#5106)	TAC CAT GCA GGG TGG AAT GT	100 µM	
	6A/C-rev (#3101)	CCA TCC TTC GAG TAT TGC	100 µM	
	6C-fwd (#5325)	CAT TTT AGT GAA GTT GGC GGT GGA GTT	100 µM	
	6C-rev (#3325)	AGC TTC GAA GCC CAT ACT CTT CAA TTA	100 µM	
Pilus PCR (Moschioni)	459_F	AAC TGA ATT GAC ACA ACG TGT CTT	100 µM	7.5 µM
	470_R	GCC ACA CAA GAT GTT GAT GCT TTT	100 µM	7.5 µM
	P01_R	AGC GAC AAG CCA CTG TAT CAT ATT	100 µM	7.5 µM
	P08_F	TGA GAT TTT CTC GTT TCT CTT AGC	100 µM	7.5 µM
	P08_R	AAT AGA CGA TGG GTA TTG ATC ATG T	100 µM	7.5 µM

Taqman probes are labelled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) at the 3'-end.

[1]Taqman probes are labelled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "T" residue with BHQ1, with a modified 3'-end to prevent probe extension by Taq polymerase.

*NLN_P working concentration was 50 μM in the original paper, however at SMRU 10 μM was better when using a 10OD Proligo probe stock (ordered in late 2010): 50 μM resulted in non-specific fluorescence in all tubes.

13 Appendix 3: Publications resulting from this thesis

13.1. Work described in the thesis

Turner P, Turner C, Jankhot A, Helen N, Lee SJ, Day NP, et al. A longitudinal study of *Streptococcus pneumoniae* carriage in a cohort of infants and their mothers on the Thailand-Myanmar border. PLoS One. 2012;7(5):e38271.

Turner P, Hinds J, Turner C, Jankhot A, Gould K, Bentley SD, et al. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol. 2011 May;49(5):1784-9.

Turner P, Melchiorre S, Moschioni M, Barocchi MA, Turner C, Watthanaworawit W, et al. Assessment of *Streptococcus pneumoniae* pilus islet-1 prevalence in carried and transmitted isolates from mother-infant pairs on the Thailand-Burma border. Clin Microbiol Infect. 2012;18(10):970-5.

13.2. Work resulting from the cohort study, but not included in the thesis

Turner P, Turner C, Kaewcharernnet N, Mon NY, Goldblatt D, Nosten F. A prospective study of urinary pneumococcal antigen detection in healthy Karen mothers with high rates of pneumococcal nasopharyngeal carriage. BMC Infect Dis. 2011;11:108.

Turner P, Po L, Turner C, Goldblatt D, Nosten F. Detection of respiratory viruses by PCR assay of nasopharyngeal swabs stored in skim milk-tryptone-glucose-glycerol transport medium. J Clin Microbiol. 2011 Jun;49(6):2311-3.